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Seurat-1: HepaRG, repeated and single dose exposure for Mitochondrial Health and LipidTox

Elisabeth Joossens

Gilles Bories

Thomas Cole

Salvador Fortaner Torrent

Jean-Michel Gineste

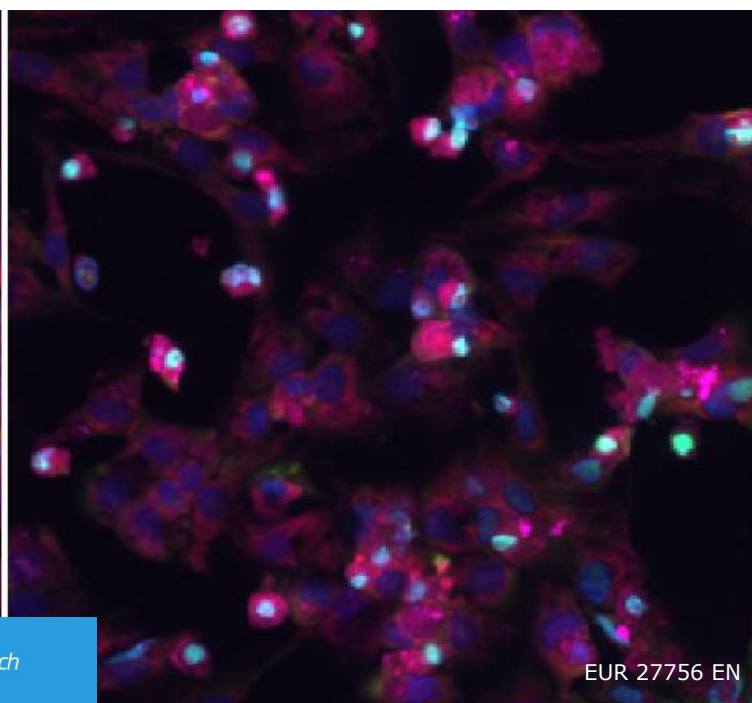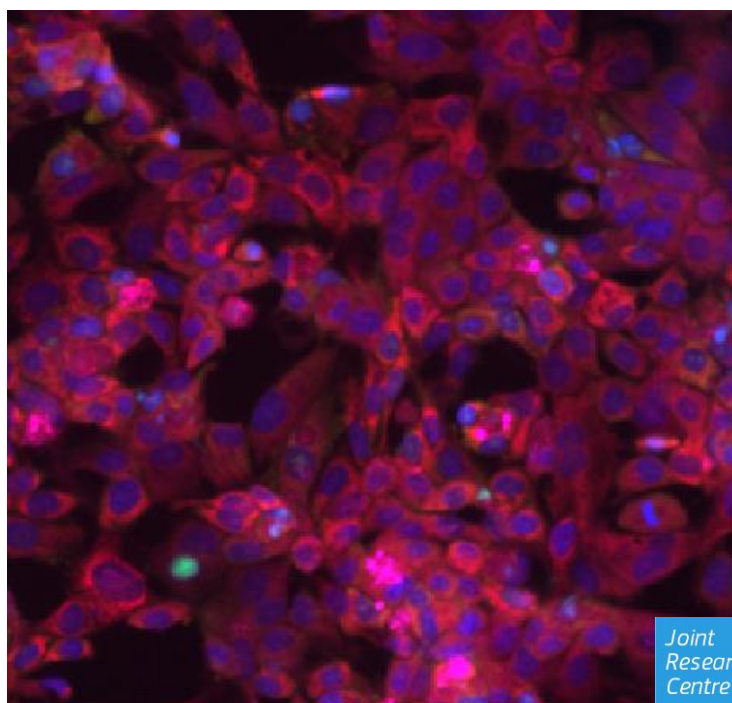Tomislav Horvat

David Canovas Jorda

Alfonso Lostia

Peter Macko

Jaroslav Novak

Taina Palosaari

Maurice Whelan

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Contact information Name: Elisabeth Joossens
Address: Joint Research Centre, Via E. Fermi 2749, I-21027 ISPRA (VA), ITALY
E-mail: elisabeth.joossens@ec.europa.eu
Tel.: +39 0332 78 6785

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Abstract

The purpose of this report is to describe the experimental procedure used in order to detect changes in mitochondrial membrane potential and lipid accumulation following exposure of HepaRG cells to various chemicals both by repeated exposure as single exposure to chemicals. This procedure was created for the SEURAT-1 Project and was developed by using live cell imaging.

1. Introduction – Purpose and applicability

This report describes research activities performed within the Seurat-1 Research Initiative launched by Cosmetics Europe and the European Commission with the long-term goal of achieving "Safety Evaluation Ultimately Replacing Animal Testing". More specifically, it is part of a SEURAT-1 Level 2 proof-of-concept (PoC) case study, formalized with a research contract between Cosmetics Europe and the European Commission's Joint Research Centre (Contract No. 32485-COLIPA-2011-T1CD ISP).

It is a follow up of deliverable 1.3 (describing a practical feasibility study aimed to predict repeated dose liver toxicity and describing the research strategy and Mode of Action(MoA)-based reasoning process used to design the study) and deliverable 3.2 (a description of a workflow, including automated protocols, used to acquire concentration-response data) as it contains the detailed 'cookbook' of the data produced within this case study.

Human primary hepatocytes are a widely used model to study Cytochrome P450 (CYP)-mediated metabolism and induction following exposure to new drugs in humans. However, their usage is limited by the restricted availability of liver tissue, their early phenotypic changes and their limited life-span. Furthermore, primary hepatocytes exhibit large variability at the level of CYP activities and the magnitude of induction after incubation with prototypical inducers - often greater than seen in in vivo situation (Guillouzo et al., 1993). The human hepatoma cell line HepaRG displays hepatocyte-like functions and expresses drug detoxifying enzymes at a relatively high levels compared to cell lines like HepG2 (Kanebratt et Andersson, 2008), drug transporter proteins and nuclear receptors. HepaRG cells were obtained from a liver tumor of a female patient suffering from hepatocarcinoma and hepatitis C infection (Gripon et al., 2002). Undifferentiated bipotent proliferating HepaRG progenitor cells start to differentiate toward hepatocyte-like and biliary-like cells at confluence. If hepatocyte-like cells are selectively isolated and cultured at high cell density, they proliferate only slightly, while still preserving their differentiation status. However, when plated at low density, they transdifferentiate into hepatocyte and biliary lineages through a bipotent progenitor stage (Cerec et al., 2007).

Liver Adverse Outcome Pathways (AOPs) have been used to select the most relevant assays available for the HTS platform. As part the project, knowledge regarding molecular pathways leading to development of liver fibrosis, steatosis and cholestasis was used to carefully select the endpoints to be tested. These endpoints reflect either general liver - and other organs - toxic effects of compounds (cytotoxicity, mitochondrial damage, apoptosis) or more specific ones, such as accumulation of lipid droplets (steatosis).

The purpose of this report is to describe the experimental procedure used in order to detect changes in mitochondrial membrane potential and lipid accumulation following exposure of HepaRG cells to various chemicals both by repeated exposure as well as single exposure to chemicals. This procedure was created for the SEURAT-1 Project runs and was developed by using live cell imaging (details of the runs see Annex 1). The report will be complemented with the final deliverable, which will contain the final conclusions one can draw from the produced data.

2. Definitions

SOP	Standard Operating Procedure
PTP	Prototype Testing Platform
RTP	Routine Test Platform
HTS	High Throughput Screening
HCI	High Content Imaging
CT	Chemical Tube
TP	Test Plate
MP	Mother Plate
DP	Daughter/Deepwell Plate
ROS	Reactive Oxygen Species
DHE	Dihydroethidium
DMSO	Dimethyl sulfoxide
PBS	Phosphate Buffered Saline

3. Principle of the Procedure

This procedure describes the steps involved in culturing, maintenance, automated seeding, chemical preparation and exposure of HepaRG cells, using the robotic platform and High Content Imaging (HCI) and a suggestion for data analysis. The following Figure summarizes the procedure.
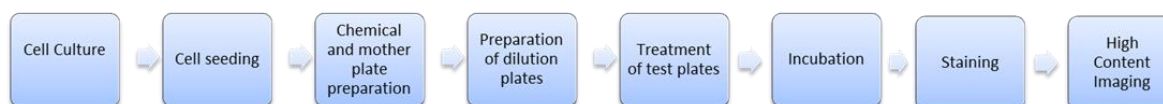
Figure 1: Steps involved in the SOP for culturing, maintenance, automated seeding and treatment of HepaRG cells with chemicals, followed by a staining and automated imaging procedure.

4. Materials and Reagents

4.1 Cells

The HepaRG cell line was established by INSERM (National Institute of Health and Medical Research) laboratory at Rennes, France. The cell line is patented by INSERM. HepaRG cells were obtained from INSERM under the material transfer agreement (N. CCR.IHCP.CA31029) for the scientific project: "Hepatotoxicity profiling of chemical substances using HepaRG model". Cells were provided by BIOPREDIC International at passage number 13.

Cells are stored at the Liquid Nitrogen container in building 101, room 061.

The inventory can be found at U:-HTS lab-Quality-HTS Cell

4.2 Instruments

4.2.1 Prototype Testing Platform (PTP)

The PTP Platform consists of two main parts, Microlab STARlet and Microlab STAR, as illustrated in Figure 1. The STARlet platform is used for cell seeding (section 5.1.5.) following the deck layout in Figure 3.

4.2.2 Routine Test Platform (RTP)

For preparation of serial dilutions (see section 5.3.2) as well as for plate treatment (see section 5.3.4). The STAR Platform with the robotic arm performs the movements of 96-well plate from and to the incubator, removes and puts back the lid and moves the plate to and from the Starlet Platform.

The PC of the Star Platform is further referred to as 'Big' and the PC of the Starlet as 'Small'.
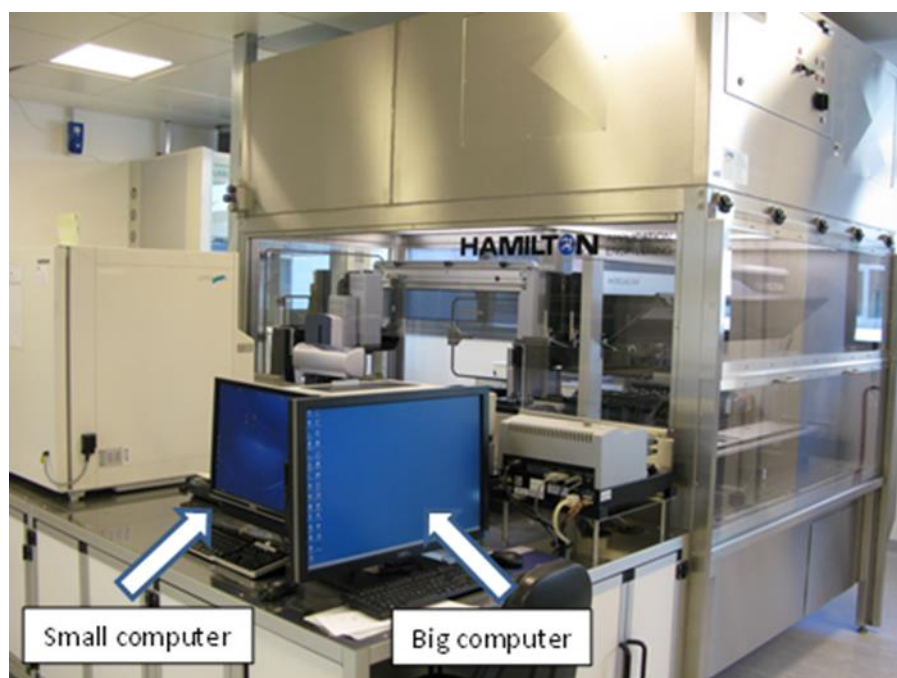
Figure 2: RTP Platform

4.3 Technical equipment

Cell culture:

- Haemocytometer (Bürker chamber or equivalent)
- Freezer at -20°C
- Laminar flow hood (Class II)
- Optical inverted microscope
- Refrigerator at +4°C
- Water bath
- Humidified cell culture incubator 5% CO_2 at +37°C
- Vortex mixer
- Pipettes and sterile tips
- NALGENE Mr Frosty Freezing Container
- Liquid Nitrogen container

4.4 Reagents, medium, serum Laboratory consumables

Reagents
- Dimethyl sulfoxide (DMSO) (Sigma, Cat. No. D2650)
- PBS Ca2+ and Mg2+ free (Gibco, Cat. No. 20012)
- Hydrocortisone (Sigma, Cat. No. H0888)
- Insulin (Sigma, Cat. No. I6634)
- L-Glutamine 200 mM (Gibco, Cat. No. 25030-024)
- Penicillin Streptomycin (PenStrep) (Gibco, Cat. No. 15140-122)
- Trypsin-EDTA 0.5%[1] (Gibco, Cat. No. 15400-054)
- Trypan Blue (Sigma, Cat. No. T8154)
- Hydrochloric acid, 37% (Sigma, Cat. No. 258148)
- Ethanol (Sigma, Cat. No. 02854)

Cell Culture Medium
- William's MediumE (Gibco Cat. No. 22551-022)

Serum for cell maintenance
- HyClone Fetal Clone III, US Origin (HyClone Cat. No. SH30109.03)

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[1] Aliquot 0.5% trypsin in 1ml aliquots and store at -20°C. Add 9 mL of PBS - Ca^{2+}/Mg^{2+} free- to 1 mL of 0.5% trypsin for a final trypsin working concentration of 0.05%.

Cell culture plastic ware and disposables:

- 75 cm^2 cell culture flasks (Corning, Cat. No. 430720)

- 150 cm^2 cell culture flasks (Corning, Cat. No. 430825)

- Conical plastic tubes, 15 and 50 ml, and suitable racks (Falcon, Becton Dickinson Cat. No. 352097 and 352098 respectively)

- 20, 200 and 1000 μl, sterile pipette tips (Eppendorf, Cat. No. 022491067, 022491083 and 022491105 resp.).

- Sterile Eppendorf tubes (Eppendorf, Cat No: 0030120086)

- 5, 10 and 25 ml Pipettes, plastic (Falcon, Cat. No. 357543, 357551 and 357525 resp.)

- Syringes, sterile 75-90 ml (PIC, Cat. No. 0373)

- Filters, sterile 0.22 μm (Millipore, Cat. No. SLGP033RS)

- 96-well clear bottom black polystyrene microplates (Costar, Cat. No. 3904)

- Clear 96-well plates (Falcon cat. No 3702)

- Hamilton tips (CORE tips 300 μl)

- Sterile plastic Matrix reservoir for cells seeding (96 deepwell SIZE: 20) (ThermoScientific, Cat. No. 1064-05-6)

- Sterile Deep well plates for serial dilutions (Eppendorf, Cat. No. H 0030506 308)

- Matrix 1ml 2D barcoded glass tubes racks of 96 tubes/case (Twin Helix,Cat.No. 3850)

- Corning Cryogenic Vials (Cat. No. 430488)

4.5 Material Prepartion

[Note: all procedures must be carried out under aseptic conditions and in the sterile environment of a laminar flow cabinet (class II biological hazard standard). All methods and procedures must be adequately documented.]

Complete medium is stable for 14 days.

Serum

1.   Thaw the serum bottle slowly at 4oC for approximately 48 h.
2.   Prepare 50 ml serum aliquots and store at -20oC.
3.   Final concentration of serum in the culture medium should be of 10% (v/v), (e.g. Add 50 ml of serum on a 500 ml culture medium final volume).

PenStrep solution

1.   Thaw the 100 ml PenStrep bottle at 40C
2.   Prepare 5 ml aliquots and store at -200C.
3.   Final concentration of PenStrep in the culture medium should be of 1% (v/v), (e.g. Add 5 ml of PenStrep solution on a 500 ml culture medium final volume).

Insulin solution

1.   Prepare an Insulin stock solution of 10 mg/ml in PBS acidified by hydrochloric acid (HCL); stock solution can be maintained at 40C for 2 months.
2.   Stock solution can be prepared as followed: dissolve 39 mg of Insulin powder in 4 ml of PBS, add 20 µl of 37% HCL and adjust the volume with PBS to 5 ml final. Filter sterilize.
3.   Final concentration of Insulin in the culture medium should be of 5 µg/ml (e.g. add 0.32 ml of Insulin stock solution on a 500 ml culture medium final volume).

Hydrocortisone solution

1.   Prepare a Hydrocortisone stock solution of 29 mM in Ethanol; stock solution can be maintained at 4°C for 2 months.
2.   Stock solution can be prepared as followed: dissolve 105 mg of Hydrocortisone powder in 10 ml of Ethanol. Filter sterilize.
3.   Final concentration of Hydrocortisone in the culture medium should be of 50 µM (e.g. add 0.863 ml of Hydrocortisone stock solution on a 500 ml culture medium final volume).

L-glutamine

1.   Thaw the 100 ml L-glutamine bottle at room temperature for several hours.
2.   Prepare 5 ml aliquots and store at -20oC.
3.   When thawing, avoid keeping the L-Glutamine for too long in +37°C and mix by vortexing for a minimum time necessary.
4.   Final concentration of L-glutamine in the culture medium should be of 2 mM, (e.g. Add 5 ml of L-glutamine solution on a 500 ml culture medium final volume).

DMSO

DMSO is added only in the differentiation culture medium.

1.   DMSO (and DMSO containing reagents) should be kept at room temperature and protected from light.
2.   Final concentration of DMSO in differentiation culture medium should be 1.7% (v/v), (e.g. add 8.5 ml of DMSO on a 500 ml culture medium final volume). Note, that the day before the differentiation will start, the cells are exposed to 0.85% DMSO.

Table 1:Resume

Compound	Final concentration	Stock solution concentration	mL (e.g. 500mL final)		Culture medium	
William's E without L-glutamine	-	-	438,82	430.32	Maintenance media	Differentiation media
Serum	10%	-	50	50		
PenStrep	1%	10.000U Penicillin 10.000mg Streptomycin	5	5		
Insulin	5 µg/mL	10 mg/ml	0.320	0,320		
Hydrocortisone	50 µM	29mM	0.863	0.863		
L-glutamine	2 mM	200 mM	5	5		
DMSO	1.7%	-	-	8.5		

5. Methods

5.1 Cell Maintenance and culture procedures

Undifferentiated HepaRG cells were provided by Biopredic International in cryopreserved vials with 1×10^6 cells/ml at passage number 13 (Biopredic, Rennes, France, Cat. No. HPR101).

[Note: the HepaRG cells cannot be passaged more than 20 times. At passage 21 the cells start to go to senescence.]

[Note2: Warm medium, PBS and trypsin before its use]

5.1.1 Cell thawing

1. Thaw cells by transferring the vial from liquid nitrogen rapidly to a +37°C water bath.

2.a.) Re-suspend the cells in 3-4 ml of fresh, pre warmed, maintenance culture medium in a 75 cm^2 flask and add maintenance medium to a final   volume of 12 ml.

<div align="center">OR</div>

   b.) Move the cells from the cryovial to a 50 ml sterile Falcon tube and add 3-4 ml of pre-warmed maintenance medium very slowly drop wise on the cells to avoid osmotic shock. Transfer the cell suspension to a 75 cm2 flask and add maintenance medium to a final volume of 12 ml.

3. Keep the cell cultures in a humidified CO_2 (5 % in air) incubator at 37°C until cell attachment (ca. 6h).

4. Once cells are attached, replace culture medium.

5. Refresh culture medium every 2-3 days (e.g. Monday, Wednesday & Friday).

6. Once cells reach confluence (eg.on day 7) they are re-seeded in a 150 cm^2 flask (see section 5.1.2).

5.1.2 Maintenance of Undifferentiated cells

1. Aspirate culture medium.

2. Wash cells with 10 ml of PBS (without Ca^{2+} and Mg^{2+}).

3. Add 2 ml of warm 0.05% Trypsin-EDTA.

4. Place flask at 37°C for 3-4 minutes. Control under the microscope to verify complete cell detachment.

5. Suspend cells in 5 ml of fresh culture medium and collect them. Flush the flask with 10 ml of fresh culture medium and add it to the suspended cells. Eliminate cell clumps by vigorous pipetting of cell suspension.

6. Count cells (see section 5.1.3).

7. Seed 4×10^6 cells per 150 cm^2 flask (final volume of cell suspension 20 ml)

8. Incubate the cells in a humidified CO_2 (5 % in air) incubator at 37°C.

9. Refresh culture medium every 2-3 days (e.g. Monday, Wednesday & Friday) for 13 days.

5.1.3 Cell counting

1. Add 20 µl of cell suspension to 20 µl of Trypan Blue dye (1:2).

2. Use a haemocytometer (eg. Bürker) to calculate the number of cells per ml.
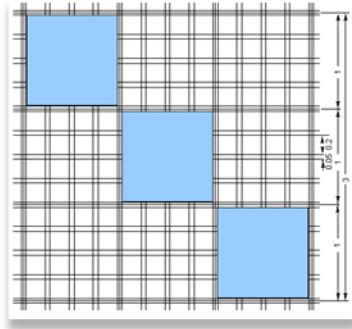
Average number of cells counted (in colored fields) x 2 (dilution factor) x 1×10^4= number of cells in 1 ml suspension

5.1.4 Cell differentation

1. Keep the cells (seeded at 4×10^6/flask 150 cm^2) in the maintenance medium for 13 days.

2. On day 13, when the cells have reached confluence, replace culture medium by differentiation medium (maintenance medium supplemented with 0.85% of DMSO).

3. On the following day, remove medium and add 20 ml of differentiation medium containing 1,7% of DMSO.

4. Exchange the medium every 2-3 days (e.g. Monday, Wednesday & Friday) with differentiation medium containing 1.7% of DMSO for a total of 14 days.

5.1.5 Cell seeding

Trypsinize cells from a 150 cm2 flask following the described steps:

1.   Aspirate culture medium.

2.   Wash cells with 10 ml PBS (-Mg^{2+}, -Ca^{2+}) for 1 min.

3.   Remove PBS.

4.   Add 4 ml of warm 0.05% trypsin-EDTA for a few seconds.

5.   Remove 2 ml of the trypsin-EDTA, ensure that left/remaining trypsin is homogenously distributed throughout the flask surface.

6.   Place the 150 cm^2 flask at 37 °C for 4 minutes. Have a look at the microscope to follow selective hepatocyte detachment. Gentle flask shaking may help selective detachment.

7.   Re-suspend cells in 5 ml of fresh maintenance culture medium and collect them. Wash the 150 cm^2 flask with 10 ml of fresh culture medium and add it to the resuspended cell suspension.

8.   Use a 10 ml pipette to disrupt cell clumps and obtain a homogeneous cell suspension.

9.   Count the cells (see section 5.1.3).

		Corning 96-well plate cod.3904	Corning 96-well plate cod.3904	Corning 96-well plate cod.3904	
		Corning 96-well plate cod.3904	Corning 96-well plate cod.3904	Corning 96-well plate cod.3904	
	Tips Rack (300 µL)	Corning 96-well plate cod.3904	Corning 96-well plate cod.3904		
		Corning 96-well plate cod.3904	Corning 96-well plate cod.3904		Lid Park
		Corning 96-well plate cod.3904	Corning 96-well plate cod.3904		Cell suspension container

Figure 3: Deck layout for Cell Seeding on Hamilton Starlet Platform on PTP

Before seeding the cells, resuspend them thoroughly with a pipette to remove eventual cell aggregates.

Seed the cells at $5x10^4$ cells per well (100 µl) in a 96-well plate (high density) using the Microlab STARlet platform:

Switch on the laminar flow, clean the cell seeding platform liquid handling workstation (Hamilton Microlab STARlet on PTP) before the seedingprocess.

Place 12 sterile 96 wells plates (Costar, Catalog No 3904), tips (CORE Tips 300µl) and sterile Matrix reservoir (ThermoScientific) with appropriate volume of cell suspension as indicated in.Figure 3.

In the slave PC select 'Hamilton'-Methods'-'NanoMILE'-'Nano seeding' Select the number of plates to be seeded (max.15, 12 needed for Seurat experiment)

RUN Method

Incubate the plates in the Cell Culture Lab for 72h at +37°C, 5% CO_2 before exposure.

5.1.6 Freezing of undifferentiated cells

1.) Culture the cells until they reach confluency.

2.) Once confluency is reached, 24 h. before freezing replace cell culture media with fresh undifferentiation media.

3.) Detach the cells following the subculturing procedure as described in section 5.1.2 (steps 1 to 4).

4.) Transfer cell suspension to a 15 ml Falcon tube and centrifuge for 2 minutes at 1500 rpm. Re-suspend the cells in 4-5 ml of cold undifferentiation

medium. Homogenise the suspension and count the cells. Adjust the volume to get $2x10^6$ cells/ml. Place the suspension on ice.

5.) Distribute 500 µl of cell suspension in 1.8 ml cryotubes maintained on ice.

6.) Then, use cold Freezing Medium composed of undifferentiation medium 10% (v/v) FBS and with 20% (v/v) DMSO.

7.) Add 500 µl of the Freezing Medium slowly to the cell suspension in cryotubes in order to obtain 1 ml of cell suspension with $1x10^6$ cells in medium with final DMSO concentration of 10%. Maintain cryotubes on ice.

8.) Place the cryotubes in a NALGENE Mr Frosty freezing container filled with with 100% isopropyl alcohol (rate of cooling 1°C/min.), place the containers in a freezer at -80°C until the next day, when the will be transferred to a liquid nitrogen container.

9.) Update the cell inventory records.

5.2 Chemical preparation

5.2.1 Selection

The selection was governed by the availability of supporting data from multiple literature sources. The test set comprises 90 reference chemicals with 75:25 % positive: negative. Positive reference chemicals must have clear and robust evidence that they cause hepatotoxicity based on in vivo data. Furthermore, some of them should also be listed in TG-GATES and/or ToxCast databases in order to facilitate further data analysis and comparison.

From literature searching, a list of pharmaceuticals known to cause hepatotoxicity based on human data was compiled. Particularly, following references were used:

– O'Brien et al., Arch Toxicol 2006;80:580–604

– Xu JJ et al., Toxicological Sciences 2008;105:97–105

– Gómez-Lechón MJ et al, Toxicology in Vitro 2010;24:1879–1889

– Poreceddu et al., Toxicol Sci 2012;129:332-45

– Gustafsson F et al., Toxicol Sci 2014;137:189-211

– Garside H et al., Toxicol In Vitro 2014;28:171-81

This list of chemicals was then expanded to include compounds from the SEURAT-1-Gold Compound list (particularly 8 chemicals are included) COSMOS DB, ToxRef DB and the Hazard Evaluation Support System (HESS) database. The list also includes chemicals for which PBTK models are being developed within COSMOS to perform in vitro to in vivo extrapolation. Negative reference chemicals were also selected from the above-mentioned sources. For all reference compounds, the chemical diversity is explored (i.e. selecting structurally diverse positives and negatives).

The final list of selected chemicals can be found in Annex 2.

5.2.2 Solubility analysis by nephelometry

Poor compound solubility can significantly affect interpretation of results obtained in toxicological studies (e.g. the precipitate can interfere with cellular transport across the membranes). Usually, the solubility of a chemical in DMSO/medium is determined by

visual inspection, which is a very imprecise method. An alternative is to perform the nephelometric analysis, which is based on measuring relative turbidity of fluid suspensions and colloids. Turbidity, indicative of chemical insolubility, is quantified as Tyndall effect light scattering from a transmitted laser beam, proportional to levels of dispersed particulate material. This is in particular useful at threshold concentration ranges of solubility where visual distinction of suspension from solution becomes subjective. Concentration ranges for tested compounds were therefore adjusted to the results of nephelometric analysis performed in both DMSO and medium.

Procedure started by adjusting the highest concentration of a compound in DMSO to 250 mM. If by visual inspection precipitation was observed (following also a centrifugation step to observe eventual precipitate formation at the bottom of a tube), serial dilutions in DMSO (dilution factor 3) were prepared until precipitate was no longer observed. This concentration was then used as the highest stock solution to be tested by nephelometer ($C1_{DMSO}$). Two additional serial dilution steps were performed (dilution step 3) and also tested by nephelometer ($C2_{DMSO}$ and $C3_{DMSO}$).

All three stock solutions in DMSO, determined as homogenous by visual inspection, were then diluted 1:1000 in undifferentiating medium (working solutions) and checked for solubility by nephelometric analysis ($C1_{medium}$, $C2_{medium}$ and $C3_{medium}$). To determine the level of insolubility, standard formazin solutions with 5 NTU and 10 NTU (nephelometric turbidity units) were used. All the prepared chemical solutions were dispensed in 96-well plates (Costar type 3599) according to the layout below (Figure 4 Measurements were performed using the NEPHELOstar Galaxy (BMG LABTECH) micro-plate nephelometer. The instrument software provides options for measurement definition and plate layout, linked to Excel® templates for systematic compilation and evaluation of results. However, for relevant statistical analysis and graphical presentation, raw data were exported to independent Excel® files. Mean values with corresponding standard deviations were calculated based on three measurements per each compound and concentration. Mean values and standard deviations for the blank and formazin solutions were based on eight measurements (following the layout in Figure 4).

Solutions with turbidity level higher than the one of 10 NTU-standard were regarded as insoluble, which necessitated lowering concentrations of both stock and working solutions. For each chemical, the highest soluble concentration (used for the testing) is recorded in Annex 2.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Solvent/ Medium Blank	Reference Item conc^n. 1	Reference Item conc^n. 2	Test Item 1(1)	Test Item 1(2)	Test Item 1(3)	Test Item 2(1)	Test Item 2(2)	Test Item 2(3)	Test Item 3(1)	Test Item 3(2)	Test Item 3(3)
B	Solvent/ Medium Blank	Reference Item conc^n. 1	Reference Item conc^n. 2	Test Item 4(1)	Test Item 4(2)	Test Item 4(3)	Test Item 5(1)	Test Item 5(2)	Test Item 5(3)	Test Item 6(1)	Test Item 6(2)	Test Item 6(3)
C	Solvent/ Medium Blank	Reference Item conc^n. 1	Reference Item conc^n. 2	Test Item 7(1)	Test Item 7(2)	Test Item 7(3)	Test Item 8(1)	Test Item 8(2)	Test Item 8(3)	Test Item 9(1)	Test Item 9(2)	Test Item 9(3)
D	Solvent/ Medium Blank	Reference Item conc^n. 1	Reference Item conc^n. 2	Test Item 10(1)	Test Item 10(2)	Test Item 10(3)	Test Item 11(1)	Test Item 11(2)	Test Item 11(3)	Test Item 12(1)	Test Item 12(2)	Test Item 12(3)
E	Solvent/ Medium Blank	Reference Item conc^n. 1	Reference Item conc^n. 2	Test Item 13(1)	Test Item 13(2)	Test Item 13(3)	Test Item 14(1)	Test Item 14(2)	Test Item 14(3)	Test Item 15(1)	Test Item 15(2)	Test Item 15(3)
F	Solvent/ Medium Blank	Reference Item conc^n. 1	Reference Item conc^n. 2	Test Item 16(1)	Test Item 16(2)	Test Item 16(3)	Test Item 17(1)	Test Item 17(2)	Test Item 17(3)	Test Item 18(1)	Test Item 18(2)	Test Item 18(3)
G	Solvent/ Medium Blank	Reference Item conc^n. 1	Reference Item conc^n. 2	Test Item 19(1)	Test Item 19(2)	Test Item 19(3)	Test Item 20(1)	Test Item 20(2)	Test Item 20(3)	Test Item 21(1)	Test Item 21(2)	Test Item 21(3)
H	Solvent/ Medium Blank	Reference Item conc^n. 1	Reference Item conc^n. 2	Test Item 22(1)	Test Item 22(2)	Test Item 22(3)	Test Item 23(1)	Test Item 23(2)	Test Item 23(3)	Test Item 24(1)	Test Item 24(2)	Test Item 24(3)

Figure 4: Plate layout for analysing solubility of 24 compounds by nephelometric analysis. Solubility of each compound is analysed in triplicate.

5.2.3 Solution Preparation

The chemicals were stored as aliquots in 100% DMSO at – 20°C. These aliquots were used for preparation of so-called "mother" plates with 90 chemicals and 1 or 2 endpoint positive controls and 4 negative controls (0.1% DMSO). A mother plate is a rack of aliquots containing the stock solution which is generally used in high throughput screening (HTS) facilities (see section 5.3 Cell Treatment). It serves as the basis for the automatic preparation of test chemical solutions used for cell treatment. In each run an additional control plate was prepared containing only negative and endpoint positive control(s) in order to obtain concentration response curves regarding these control compounds (see Figure 10).

5.3 Cell treatment using HTS platform

The summary of experimental layout, including the list of endpoint-positive controls, is presented below (Table 2).

Table 2: Experimental layout

Number of chemicals tested	90
Plate format used for experiment	96-well plate
***In vitro* cell model**	HepaRG cells
Number concentrations/chemical tested in vitro	11 (serial dilutions prepared with dilution factor 1:3)
Highest tested concentration in DMSO	250 mM; for each compound defined by nephelometric analysis
***In vitro* endpoints measured**	Cell viability, mitochondrial toxicity, lipid droplets formation
Endpoint-positive controls	Cell viability (Dead Green) - Valinomycin 7.2 µM Mitochondrial toxicity – Valinomycin 165 nM Lipid droplets formation – Cyclosporine A 30 µM
Endpoint-negative control	DMSO 0.1 % (added to four wells in every 96-well plate)
Exposure scheme	Exposure for 24 h; exposing cells to chemicals every 6 h

Well-plates used in HTS platform

–   Mother plate (96-wells):
Quantity: 1

The plates are filled with 96 bar-coded glass tubes (90 compounds + 2 positive controls + 4 negative controls), each of these containing the highest chemical stock solution in DMSO (250 mM or less depending on solubility), except for the control samples.

–   Daughter plate or dilution plate (384-wells):
Quantity: 3

The plates are used to prepare the 10 serial stock chemical concentrations in DMSO. Furthermore, the highest chemical stock solution in DMSO is transferred here which is then used to prepare the 10 serial concentrations. Two plates contain four serial concentrations while the third one contains three concentrations.

–   Deep-well plate (96-wells):
Quantity: 11 + 1

The plates are used to prepare the 11 serial working concentrations in cell medium. Each plate contains all the 90 reference chemicals at a certain concentration + the controls.

One plate contains only endpoint controls both positive and negative.

–   96-wells plate (for cell exposure):
Quantity: 11 + 1

The plates are used to expose HepaRGs to the chemicals. 11 plates are used for 24h exposure (or 4 or 72 h exposure). The reaming plate is used to expose HepaRGs to positive and negative endpoint-controls.

Barcodes need to added to the plates (end of plate, near column 12).

Optimization of HTS deck layout

The HTS platform can contain up to 20 plates. For this experiment, there is the need to have 96-well plates filled with serial concentrations in cell medium and 11 filled with serial concentrations in DMSO. In order to optimize the space available on the HTS platform, the serial concentrations in DMOS are prepared in three daughter 384-well plates rather than in 11 96-well plates.

Using this layout, there is sufficient space in the HTS platform to place also the 11 deep-well plates to prepare the serial chemical concentrations in cell medium.

As shown in Figure 5, in a 384-well daughter plate the space occupied by four wells is more or less the same of the one defined by one well of a 96-well plate. Therefore one daughter plate can contain up to four different concentrations.
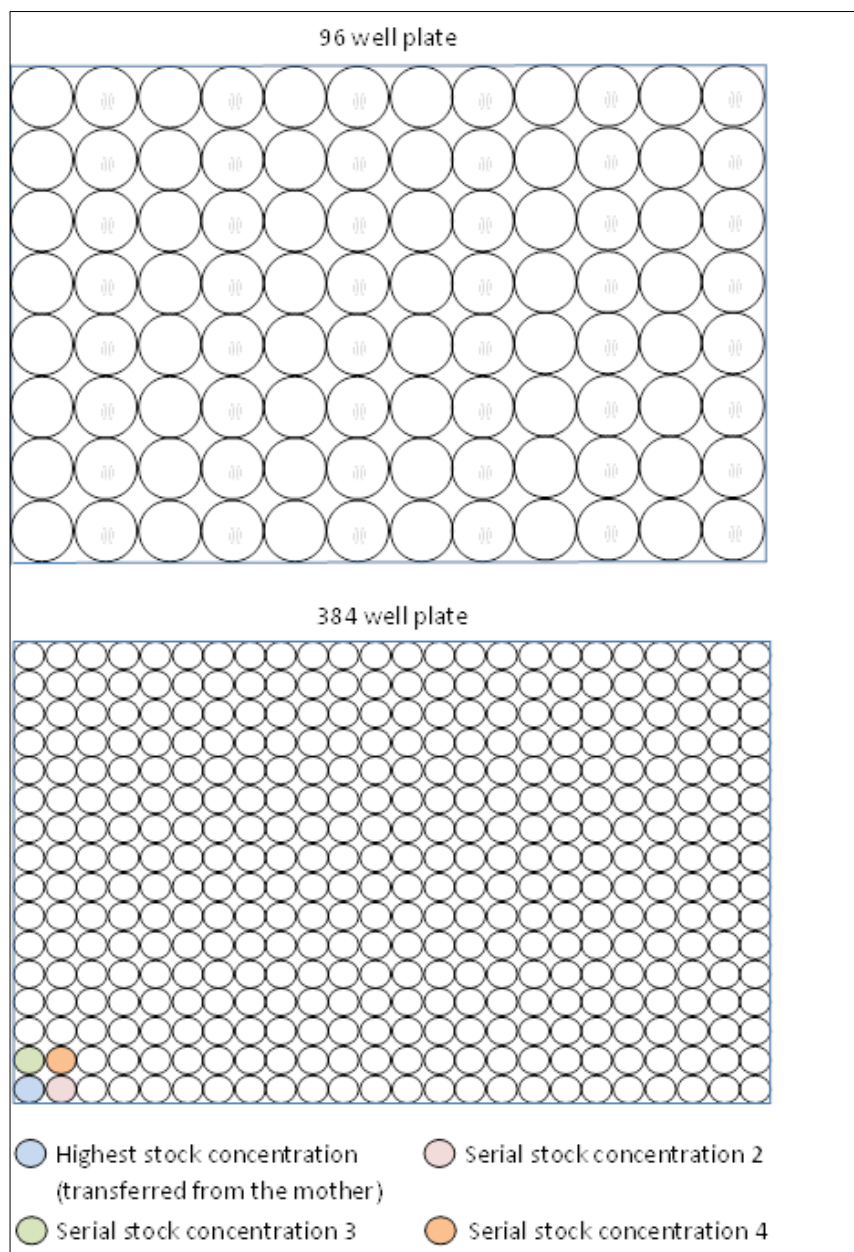

Figure 5

5.3.1 Preparation of mother plate (MP=C1)

After having defined the highest soluble concentration by nephelometric analysis, chemicals were solubilized in 100% DMSO and placed in 90 bar-coded glass tubes that together with 4 tubes containing negative controls (0.1 % DMSO) and two containing endpoint positive controls constituted a mother plate. The volume of all chemicals and controls in the glass tubes was at least 120 µl. An exemplary layout is presented in Figure 6.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	9	17	25	29	37	45	53	61	69	77	85
B	2	10	18	26	30	38	46	54	62	70	78	86
C	3	11	19	DMSO (NC)	31	39	47	55	63	71	79	87
D	4	12	20	DMSO (NC)	32	40	48	56	64	72	80	88
E	5	13	21	DMSO (NC)	33	41	49	57	65	73	81	89
F	6	14	22	DMSO (NC)	34	42	50	58	66	74	82	90
G	7	15	23	27	35	43	51	59	67	75	83	valinomycin 165 µM (PC 1)
H	8	16	24	28	36	44	52	60	68	76	84	valinomycin 20 mM (PC 2)

Figure 6: An exemplary mother plate layout showing the position of highest stock concentration (250 mM or less depending on solubility) for each of the 90 chemicals plus the position of positive and negative controls

For each run the set of 4 NC is moved randomly on the plate (with the constraint to remain together within the area B2 to G11). At the same time the compounds are randomly put on the plate.

5.3.2 HTS Preparation of chemical serial concentrations (both in DMSO and cell medium)

Step 1 - Add cell medium (1998 µL) in deep-well plates

• HTS deck layout (as shown in Figure 7)

  – Put in the left-rack, tips of 1000 µL, 300 µL, 50 µL and 10 µL
  – Put the eleven deep-well plates and the endpoint-control plate to be filled with cell medium
  – Put container with cell medium (The volume of cell medium needed to prepare all 11 deep-wells plates is 2.3 liters. Since only one container can be

used and it can contains no more than 400 mL, cell medium must be added manually to refill the container)

- HTS protocol:

  1.   Load the tips with 1 mL internal volume and select 999 µL volume to be aspirated
  2.   From the container filled with cell medium, aspirate 999 µL of cell medium, rinse the tips 3 times with cell medium and transfer to all the eleven deep-well plates the 999 µL of cell medium
  3.   Using the same tips, from the container filled with cell medium, aspirate 999 µL of cell medium and transfer them in all eleven deep-well plates. Now the total volume of cell medium is 1998 µL
  4.   Place back the tips to the corresponding Tips-Rack

Container 1	96-well plate holder	Deep well plate C 1	Deep well plate C 6	Deep well plate C 7	
Container 2	Tips Rack 1 (1 mL)	Deep well plate C 2		Deep well plate C 8	Tips Rack 1
Container 3	Tips Rack 2 (300 µL)	Deep well plate C 3		Deep well plate C 9	Tips Rack 2
Container 4	Tips Rack 3 (50 µL)	Deep well plate C 4	Deep well Endpoint Control Plate	Deep well plate C 10	Tips Rack 3
Container 5	Tips Rack 4 (10 µL)	Deep well plate C 5	Container Cell Medium	Deep well plate C 11	Tips Rack 4

Figure 7: deck layout for adding cell medium in deep-well plates

Step 2 - Add DMSO (40 µL) in 384-well plates

- HTS deck layout (as shown Figure 8)

  –   Remove the endpoint control plate to create space to place the daughter plates (then it will be put back)
  –   Put three daughter plates
  –   Put a new container with 120 mL DMSO

- HTS protocol:

  1.   Load from the tips-rack the tips (50 µL internal volume) and select 40 µL volume to be aspirated
  2.   From the container filled with DMSO, aspirate 40 µL of DMSO and transfer them in all daughter plates. In the daughter plate called S1-S3 (Figure 4) leave a set of wells empty (no addition of DMSO) to then place 40 µL from mother plate (next step) of highest chemical concentration in DMSO (S1)
  3.   Place back the tips to the corresponding tips-rack

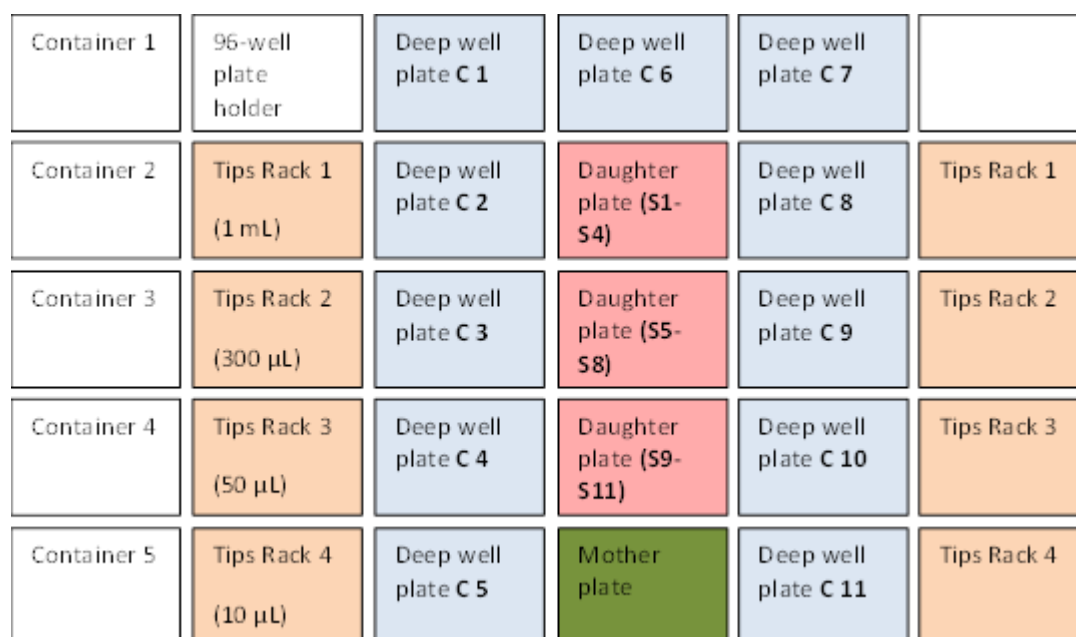| Container 1 | 96-well plate holder | Deep well plate C 1 | Deep well plate C 6 | Deep well plate C 7 | |
Container 2	Tips Rack 1 (1 mL)	Deep well plate C 2	Daughter plate (S1-S4)	Deep well plate C 8	Tips Rack 1
Container 3	Tips Rack 2 (300 µL)	Deep well plate C 3	Daughter plate (S5-S8)	Deep well plate C 9	Tips Rack 2
Container 4	Tips Rack 3 (50 µL)	Deep well plate C 4	Daughter plate (S9-S11)	Deep well plate C 10	Tips Rack 3
Container 5	Tips Rack 4 (10 µL)	Deep well plate C 5	Container DMSO	Deep well plate C 11	Tips Rack 4

Figure 8: deck layout for adding DMSO in 384-well plates

Step 3 - Prepare serial stock concentrations in DMSO (in 384-well plates)

- HTS deck layout (as shown in Figure 9)

  – Remove container with DMSO
  – Put mother plate

- HTS protocol:

  1. From the mother plate, transfer 40 µL to the top daughter-plate in the set of well left empty in Step 2. This is the S1 concentration.
  2. From the daughter plate, containing the highest concentration (S1), aspirate 20 µL of S1
  3. Transfer the 20 µL of S1 into the first daughter plate-wells already containing 40 µL of DMSO to obtain the first serial concentration (called S2)
  4. Once the 20 µL of S1 are transferred, mix[2] the solution by pipetting 50 µL
  5. Repeat the steps from 2 to 4 to prepare the remaining serial concentrations (for the runs 15 and 16 this was done using the same tips, from run 17 tips are changed every time). This time the 20 µL of S2 are used to prepare the following serial concentration (S3) and this will be performed for all the remaining serial concentrations to be prepared as described in Table 3.

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[2] Depending on program versions, mixing steps are slightly different between runs 15-16 and the remaining. For the original first version 20ul are mixed 20 times for each DMSO dilution steps. A second version fully operational in August 2015 is mixing 60 times with an increased volume of 40ul. The difference is in the value of the effective dilution factor that is really obtained at the end of the process. This changes has been introduced to improve the reduce difference between the theoretical and real dilution factor which have their origin linked with DMSO liquid properties.

Container 1	96-well plate holder	Deep well plate C 1	Deep well plate C 6	Deep well plate C 7	
Container 2	Tips Rack 1 (1 mL)	Deep well plate C 2	Daughter plate (S1-S4)	Deep well plate C 8	Tips Rack 1
Container 3	Tips Rack 2 (300 µL)	Deep well plate C 3	Daughter plate (S5-S8)	Deep well plate C 9	Tips Rack 2
Container 4	Tips Rack 3 (50 µL)	Deep well plate C 4	Daughter plate (S9-S11)	Deep well plate C 10	Tips Rack 3
Container 5	Tips Rack 4 (10 µL)	Deep well plate C 5	Mother plate	Deep well plate C 11	Tips Rack 4

Figure 9: deck layout for the preparation of serial stock concentrations

Table 3

Concentration to be prepared	Volume to be prepared		Volume used of more concentrated solution		Volume of DMSO added	Volume left after preparing more diluted concentration
S 2	60 µL		20 µL $_{of\ S\ 1}$		40 µL	40 µL
S 3	60 µL		20 µL $_{of\ S\ 2}$		40 µL	40 µL
S 4	60 µL		20 µL $_{of\ S\ 3}$		40 µL	40 µL
S 5	60 µL		20 µL $_{of\ S\ 4}$		40 µL	40 µL
S 6	60 µL	=	20 µL $_{of\ S\ 5}$	+	40 µL	40 µL
S 7	60 µL		20 µL $_{of\ S\ 6}$		40 µL	40 µL
S 8	60 µL		20 µL $_{of\ S\ 7}$		40 µL	40 µL
S 9	60 µL		20 µL $_{of\ S\ 8}$		40 µL	40 µL
S 10	60 µL		20 µL $_{of\ S\ 9}$		40 µL	40 µL
S 11	60 µL		20 µL $_{of\ S\ 10}$		40 µL	60 µL

Step 4 -Prepare serial concentrations in cell medium (in deep-well plates)

Preparation of stock serial dilutions was followed by preparation of working serial dilutions (1:1000 dilution step in undifferentiating HepaRG medium: c1 – c11), reducing DMS0 to a non-toxic concentration of 0.1%

- HTS deck layout (as shown in Figure 9, just remove mother plate)

- HTS protocol:
    1.   Load the tips with 10 µL internal volume and select 2 µL volume to be aspirated
    2.   From the daughter plate containing the lowest stock concentration (S11), aspirate 2 µL of solution
    3.   Transfer the 2 µL of S11 into the corresponding deep-well plate, already containing 1998 µL of cell medium, to obtain the lowest working concentration (called C11)

4.   Once the 2 µL of S11 are transferred, wash the tips in the just prepared solution by pipetting 10 µL for three times

5.   Using tips with 10 µL internal volume[3], prepare the following serial working concentrations by repeating the steps 3 to 4. This time the 2 µL of S10 are used to prepare the following serial concentration (C10) and this will be performed for all the remaining serial concentrations to be prepared (see Table 4).

6.   Once all the working concentrations are prepared, place back to the rack the tips with 10 µL internal volume

7.   In order to properly mix all the concentrations just prepared across the 11 deep-well plates, load the tips with 1 mL internal volume and select 1000 µL volume to be aspirated

8.   Mix the solutions of every deep-well plate by pipetting 1000 µL for three times and starting with the plate containing the lowest concentration (C11)

Table 4

Working concentrations in cell medium to be prepared	Volume of working concentrations to be prepared in cell medium		Volume of cell medium used		Volume of stock solution in DMSO added
C 1	2 mL		1998 µL		2 µL of S1
C 2	2 mL		1998 µL		2 µL of S2
C 3	2 mL		1998 µL		2 µL of S3
C 4	2 mL		1998 µL		2 µL of S4
C 5	2 mL		1998 µL		2 µL of S5
C 6	2 mL		1998 µL		2 µL of S6
C 7	2 mL	=	1998 µL	+	2 µL of S7
C 8	2 mL		1998 µL		2 µL of S8
C 9	2 mL		1998 µL		2 µL of S9
C 10	2 mL		1998 µL		2 µL of S10
C 11	2 mL		1998 µL		2 µL of S11

5.3.2 Preparation of 96-well plate for endpoint control

An additional mother plate containing only negative and endpoint positive controls was prepared manually in every experiment. The exemplary layout containing controls for the detection of mitochondrial damage is shown in Figure 10

1.   Fill negative control wells with 80 µl of DMSO 100 %
2.   Fill columns 2-4 and 8-10, rows B-H, with 80 µl of DMSO 100 %
3.   Fill columns 2-4 and 8-10, row A, with 120 µl of appropriate endpoint positive control
4.   Transfer 40 µl of stock concentration (S1) from well in column 2, row A to well below (column 2, row B) and mix well by pipetting, in order to prepare 1:3 diluted solution (S2). Repeat the procedure until reaching row H (S8). Repeat the procedure in all columns containing endpoint positive controls.

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[3] For the first two runs the same tips are used while from August onwards tips are replaced every time.

	1	2	3	4	5	6	7	8	9	10	11	12
A	DMSO 100%	valinomycin 165 µM (PC 1) - S1			DMSO 100%	DMSO 100%	DMSO 100%	valinomycin 20 mM (PC 2) - S1			DMSO 100%	DMSO 100%
B	DMSO 100%	valinomycin (PC 1) - S2			DMSO 100%	DMSO 100%	DMSO 100%	valinomycin (PC 2) - S2			DMSO 100%	DMSO 100%
C	DMSO 100%	valinomycin (PC 1) - S3			DMSO 100%	DMSO 100%	DMSO 100%	valinomycin (PC 2) - S3			DMSO 100%	DMSO 100%
D	DMSO 100%	valinomycin (PC 1) - S4			DMSO 100%	DMSO 100%	DMSO 100%	valinomycin (PC 2) - S4			DMSO 100%	DMSO 100%
E	DMSO 100%	valinomycin (PC 1) - S5			DMSO 100%	DMSO 100%	DMSO 100%	valinomycin (PC 2) - S5			DMSO 100%	DMSO 100%
F	DMSO 100%	valinomycin (PC 1) - S6			DMSO 100%	DMSO 100%	DMSO 100%	valinomycin (PC 2) - S6			DMSO 100%	DMSO 100%
G	DMSO 100%	valinomycin (PC 1) - S7			DMSO 100%	DMSO 100%	DMSO 100%	valinomycin (PC 2) - S7			DMSO 100%	DMSO 100%
H	DMSO 100%	valinomycin (PC 1) - S8			DMSO 100%	DMSO 100%	DMSO 100%	valinomycin (PC 2) - S8			DMSO 100%	DMSO 100%

Figure 10: Mother plate layout containing negative and endpoint positive controls for detection of mitochondrial damage and cell viability (Dead Green).

- HTS deck layout (as shown in Figure 11)
    - Remove daughter plates
    - Put endpoint-control mother plate
    - Put back the deep well endpoint-control plate
    - Replace tips of 10 µL in the left rack
    - Replace tips of 1000 µL in the left rack

- HTS protocol:

    1. Load the tips with 10 µL internal volume and select 2 µL volume to be aspirated
    2. From the endpoint-control mother plate aspirate 2 µL of solution
    3. Transfer the 2 µL in the corresponding deep-well endpoint-control plate, already containing 1998 µL of cell medium
    4. Once the 2 µL are transferred, wash the tips in the just prepared solution by pipetting 10 µL for three times
    5. Place back to the rack the tips with 10 µL internal volume
    6. In order to properly mix all the concentrations just prepared, load the tips with 1 mL internal volume and select 1000 µL volume to be aspirated
   7. Mix the solutions by pipetting 1000 µL for three times

Container 1	96-well plate holder	Deep well plate C 1	Deep well plate C 6	Deep well plate C 7	
Container 2	Tips Rack 1 (1 mL)	Deep well plate C 2		Deep well plate C 8	Tips Rack 1
Container 3	Tips Rack 2 (300 µL)	Deep well plate C 3		Deep well plate C 9	Tips Rack 2
Container 4	Tips Rack 3 (50 µL)	Deep well plate C 4	Deep well enpointControl Plate	Deep well plate C 10	Tips Rack 3
Container 5	Tips Rack 4 (10 µL)	Deep well plate C 5	EndpointControl mother	Deep well plate C 11	Tips Rack 4

Figure 11: HTS deck layout for the preparation of serial concentrations in cell medium

5.3.4 Cell treatment

Since the goal was to keep the chemical concentration to which cells are exposed relatively constant over time - continuous exposure - in order to maximize the chances for obtaining an in vitro response (see internal document Deliverable D1.3 Final report of the feasibility study to demonstrate the prediction of selected types of target organ toxicity, Pubsy JRC95069) we opted for a repeated exposure scenario for the first 3 runs, currently including one time point (24h; re-exposures every 6h), followed by 3 runs with a single exposure of 24h. A short time point (4h), a longer one (72h; re-exposures every 6h) will be considered in the future.

72 hours after the seeding, HepaRG cells were exposed to 92 chemicals at 11 concentrations and incubated at 37°C, 5% CO2, 100% humidity for 24hours (Figure 12). In each plate, 4 negative control wells (cells treated with only DMSO) were included. All the treatment procedures were completely automated and the entire process was managed by Hamilton software.
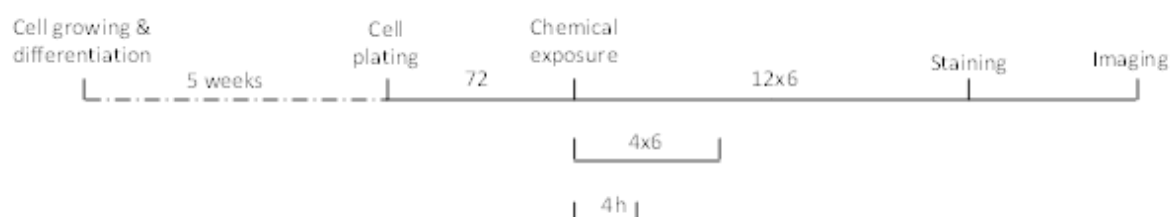
Figure 12: HepaRG culturing and treatment scheme

The following protocol is repeated according to the exposure scheme applied during the course of the experiment.

- HTS deck layout (as shown in Figure 13)

    The layout in Figure 13 is intended to replace the tips after every 6 h exposure in order to avoid cell medium dried within the tips.

    For the repeated exposure, the practical solution is that after the first exposure to cells is completed (let's say at 12), the tips are replaced manually, in the left

rack, with new ones. Then, the second exposure will be performed with new tips and as soon also this is completed (let 'say by 19) tips will be replaced again manually in the left rack. In this way also the third exposure (at around midnight) is done with new tips.

For the fourth exposure (around 6 in morning), the tips in the right rack are used. This is a cycle for 24 h overall exposure (it will be repeated two more times when a 72 h exposure is performed).

− Change all tips and put in each tip-rack (left and right), tips of 300 µL [4]
− Put a Lid-Holder
− Cover all deep-well plates (12) with a lid
− Change position of C1 deep well plate
− Put waste container

- HTS protocol:

1. From the incubator, using the robotic arm, take the first 96-wells plate with HepaRGs seeded and transfer it to the RTP in the position of "96-wells plate holder" (Figure 13)
2. Remove the lid from the first deep-well plate containing the lowest concentration
3. Load from the first tip-rack on the left the tips with 300 µL internal volume and select 85 µL volume to be aspirated
4. Aspirate 85 µL of cell medium from the 96-well plate placed in the "96-wells plate holder"
5. Discharge the 85 µL of cell medium just aspirated into the waste container
6. Load from the second tip-rack on the left the tips with 300 µL internal volume and select 85 µL volume to be aspirated
7. Using the same tips, from the deep-wells plate containing the lowest concentration in cell medium (called C11), aspirate 85 µL of cell medium and transfer them in the 96-well plate placed in the "96-wells plate holder"
8. Place back the tips to the corresponding tip-rack (the second one)
9. Cover the deep-well plate with the lid
10. Move the 96-wells plate from the "96-wells plate holder" back to the incubator
11. Repeat the steps 2 to 10 to expose the remaining 10 96-well plates to the other concentrations. This time the 85 µL of C10 are added to the corresponding 96-wells plate and this will be performed to add all the remaining serial concentrations.
12. After all 11 plates are exposed, repeat step 2 to 5 but this time use the third tip-rack on the left to remove medium from the endpoint-control plate.
13. Repeat steps 6 to 10, but this time use the fourth tip-rack on the left to expose cells to the endpoint-controls.

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[4] The first tip-rack from top (both on left- and right-side) is used only to aspirate cell medium (containing no chemicals) from 96-well plates with HepaRGs seeded that are taken from the incubator and placed to the RTP. The second tip-rack is used only to pipette working concentrations from the 11 deep-well plates. The third tip-rack is used only to aspirate cell medium (containing no chemicals) from 96-well plate with HepaRGs seeded, taken from the incubator, and that will be exposed to endpoint-controls. The fourth tip-rack is used only to pipette endpoint-controls from the corresponding deep-well plate. Because the same tips (from the second rack) are used to add the eleven chemicals working concentrations to the corresponding 96-well plates where HepaRGs are seeded, first the lowest concentration is added and then the following one to the highest concentration. In this way, there is no significant carry-over.

Waste	96-well plate holder		Deep well plate C 6	Deep well plate C 7	
Waste	Tips, 300 µL remove medium from deep well	Deep well plate C 2		Deep well plate C 8	Tips, 300 µL remove medium from deep well
Container 3	Tips, 300 µL add medium to deep well plates	Deep well plate C 3	Lid Holder	Deep well plate C 9	Tips, 300 µL remove medium from deep well
Container 4	Tips, 300 µL remove medium from control plate	Deep well plate C 4	Deep well endpointControl Plate	Deep well plate C 10	Tips, 300 µL remove medium from control plate
Container 5	Tips, 300 µL add medium to control plate	Deep well plate C 5	Deep well plate C 1	Deep well plate C 11	Tips, 300 µL remove medium from control plate

Figure 13: HTS deck layout for repeated exposure

A single exposure is obtained by simply executing manually an abort action after the first exposure done by the program. Then plate remains in the incubator to continue their incubation time as it should be with the multiple exposure program.

5.3.5 Detection of mitochondrial membrane potential and lipid droplets (Staining)

Mitochondrial Health Kit (Invitrogen, H10295) is used for evaluating depolarization of mitochondrial membrane potential, as a good indicator of mitochondrial dysfunction. In addition, this kit includes the Image-iT DEAD Green viability stain, which as a marker of membrane permeability, can potentially differentiate between healthy cells and those whose physiology due to toxic exposure has already been slightly compromised (higher sensitivity allows observing cells that are still viable, therefore attached to the surface, but undergoing changes due to toxic exposure).
The LipidTOX Deep Red neutral lipid stain (Molecular probes, H34477) has an extremely high affinity for neutral lipid droplets, appearing as indicators of chemically induced steatosis.
Both endpoints were detected in a single experimental staining procedure. The cell staining solution for detection of mitochondrial membrane potential was prepared according to the manufacturer's recommendations and used for staining the cells for 30 min at 37°C, 5% CO_2, 100% humidity. Following medium removal, cells were fixed in 4% FA for 15 min at RT and at the same time counterstained with Hoechst 33342 (10000x). After fixation, cells were washed in PBS 1x, before adding the LipidTox cell staining solution that was prepared according to the manufacturer's recommendations and used for staining the cells for 30 min at RT. Finally, before performing high content imaging (HCI), cells were stored overnight at +4°C.

5.3.5 High Content Imaging (HCI)

5.3.6.1 Cellomics ArrayScan VTI description

For imaging analysis of the 96 well plates a Cellomics ArrayScan® VTI high content imaging instrument (Thermo Scientific) designed for high-capacity automated fluorescence imaging and quantitative analysis of fixed and live cells is used. The ArrayScan VTI has a scientific grade digital camera with multiple resolution modes. The system has an automated objective changer, allowing for control over optical resolution and field of view. It uses a high intensity mercury vapour arc light source delivering a broad (350nm-700nm) illumination spectrum. The system includes a 10- position wheel for excitation filters and a 6-position turret for dichroic/emission cubes. The ArrayScan VTI includes Thermo Scientific Cellomics iDEV-High Content intelligent acquisition software.

Instructions on the use of the robotic arm can be found in Annex 4.

5.3.6.2 Overview of work flow ("Cellomics Scan")

The iDev is used for creating and modifying Assay Protocols. An Assay Protocol includes all of the settings and parameters required to scan a plate. In this case, image acquisition and image analysis were done separately. This approach allows reanalysing previously acquired images and including additional channel specific parameters, in particular useful when defining subpopulations of cells with the "event" option.

- Protocols to be selected: from "Compartmental Analysis v.4."
Bioapplication:
1) Image acquisition: TH DEADGr MitoHealth LipidTOX 4ch HRG 13M newsubp
2) Image analysis
     a) Analysis of Ch2 and Ch3 events: TH Mito DEADGreen SEURAT 3ch HepaRG
     b) Analysis of Ch3 and Ch4 events: TH DEADGr MitoHealth LipidTOX 4ch HRG 13M newsubp

The Hoechst dye has an approximate fluorescence excitation and emission maxima at 350 nm and 461 nm respectively. This nuclear stain provides a marker for ''object identification''(Channel 1 in our set up). The filter used is "XF93 HOECHST".

The Image-iT DEAD Green marker has an approximate fluorescence excitation and emission maxima at 488 nm and 515 nm respectively. Therefore, it can be detected using FITC filter set (Channel 2 in our set up) = XF93 FITC.

The MitoHealth stain has an approximate fluorescence excitation and emission maxima at 550 nm and 580 nm respectively. It can be detected using TRITC filter set (Channel 3 in our set up) = XF93 TRITC.

The LipidTOX Deep Red neutral lipid stain has an approximate fluorescence excitation and emission maxima at 637 nm and 655 nm respectively. It can be detected using Cy5 filter set (Channel 4 in our set up) = XF93 Cy5.

The objective 20x is used to acquire 10 images (fields of vision) per well.
Following features are analysed:

ValidObjectCount	Number of valid objects identified in the well (Ch1 object selection parameters)	counts	Cell count (number of nuclei)
MEAN_ObjectAreaCh1	Average of the area of all CH1 objects in the well	pixels	Average nuclear size
MEAN_ObjectAvgIntenCh1	Average of (Average intensity of all pixels within Ch1 object) of all CH1 objects in the well	intensity	Average nuclear pixel intensity
MEAN_CircAvgIntenCh2	Average of (Average intensity of all pixels within the cirk mask) of all objexts in the well	intensity	Average pixel intensity of DEAD Green in nucleus
MEAN_RingAvgIntenCh3	Average of (Average intensity of all pixels within the ring mask) of all objects in the well	intensity	Average pixel intensity of MitoHealth marker in cytoplasm (excl. nucleus) of all cells
%HIGH_RingAvgIntenCh3	Percentage of objects in the well with Feature:MEAN_RingAvgIntenCh3 above a defined level	%	Fraction of cells with high average pixel intensity of MitoHealth marker in cytoplasm (excl. nucleus)
%EventType1Objects	(NOT CircAvgIntenCh2) AND (NOT RingAvgIntenCh3)	%	Fraction of cells with altered mitochondrial membrane potential, but uncompromised cellular membrane (no DeadGreen, low intensity MitoHealth)
%EventType2Objects	CircAvgIntenCh2 AND (NOT RingAvgIntenCh3)	%	Fraction of cells with altered mitochondrial membrane potential and compromised cellular membrane (high intensity Dead Green, low intensity MitoHealth)
%EventType3Objects	(NOT CircAvgIntenCh2) AND RingAvgIntenCh3	%	Fraction of cells with unperturbed mitochondria membrane potential and uncompromised cellular membrane
MEAN_RingSpotAvgIntenCh4	Average of (Average intensity of all spot pixels within the ring mask) of all objects in the well	intensity	Average intensity of accumulated neutral lipids in cytoplasm (excl. nucleus) per cell
MEAN_RingSpotAvgAreaCh4	Average of (Average area of all spots within the ring mask) of all objects in the well	pixels	Average area of accumulated neutral lipids in cytoplasm (excl. nucleus) per cell

2nd group of Events basend on Ch3(Mitohealth) and Ch4(LipidTOX)

%EventType1Objects	(NOT RingSpotAvgIntenCh4) AND (NOT RingAvgIntenCh3)	%	fraction of non steatotic cells with low mitochondrial membrane potential
%EventType2Objects	RingSpotAvgIntenCh4 AND (NOT RingAvgIntenCh3)	%	fraction of steatotic cells with low mitochondrial membrane potential
%EventType3Objects	(NOT RingSpotAvgIntenCh4) AND RingAvgIntenCh3	%	fraction of non steatotoic cells with unperturbed mitochondrial membrane potential (healthy)

When the Assay Protocol is defined, 12 plates (C1 - C11 + control plate) are loaded at the robotic deck and according to the instructions (Annex 3.) scanned.

Use the **Scan Plate View** to control and monitor scans.

At the end of the scan open "iView" and transfer results to Excel and store the raw data in *V: I05 – Toxicity Pathways – Projects – SEURAT-1*.

5.3 Data Analysis

The raw data is provided in the following format.

- Eleven 96-well plates per experiments.
- Every plate contains a set of chemicals, 4 solvent controls (only medium with cells) and 2 positive controls (at different concentrations).
- For each experiment multiple read outs are reported.
- Data for which problems occurred at measuring (outliers) are removed.

Applied normalization

1. Currently the following normalization is applied: for each chemical, for each concentration, the notation Xc is used
2. Average of the negative controls per plate $\overline{NC_c}$ and standard deviation of all negative controls across all plates (SD_{NC}) is calculated. This results in 11 different average values of negative controls capturing the plate noise and 1 single general standard deviation capturing the variability of the noise. [5]
3. Signal to Noise Ratio (S/N) regarding each chemical and each concentration is calculated as:

$$\frac{X_c - \overline{NC_c}}{SD_{NC}}$$

By applying this normalization, plate effects are removed and, additionally, all data is harmonized in the same way, so that different readouts can directly be compared and put together on a heat map or graph.
The aim is to identify chemicals exhibiting significant (up and/or down) effects using the above normalized data for different endpoints without using a parameterized model such

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[5] An alternative could be to use the overall average, however, this way plate specific noise will not be removed. Standard deviations could also be calculated on a plate by plate basis; however, in our experiments this is not optimal as only 4 negative controls are present.

as the Hill function to obtain an EC50. So, the null-hypothesis would be that no effect was induced. Results can easily be drawn comparing data to the threshold of -3 (for downwards effect) and +3 (for upwards effect). [6]

In order to strengthen our conclusion we also put a secondary condition, which is that significant effects will exceed this threshold at least for 2 consecutive concentrations. [7] In case this condition is met, we have a solid reason to conclude (at a 95% level of confidence) that the chemical is inducing an effect (under the conditions defined by experimental setup and the assay applied).

Following the application of normalization, we look at the potency of the effect by considering the concentration and the slope at which the Signal to Noise Ratio first exceeds the threshold. This point is referred to as the first significant effect level (FSEL;Figure 14). Data of all compounds can then be summarized creating a so called heat map.
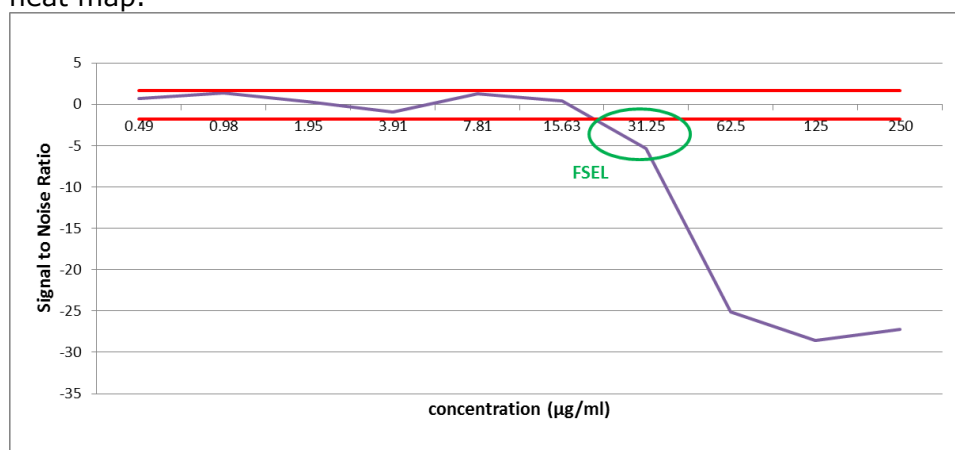
Figure 14: Example of FSEL for one feature

Quality of each run:

The quality of each run is tested by the means of the PC wells and the additional control plate with triplicates of the positive controls at different concentrations (see Figure 10). As this compound is a known control for detection of Mito damage and cell viability, we expect to see a significant effect for those channels at the relevant concentrations. We also check that the Dose response curve obtained from the plates overlap.

Position on plate effect:

In some runs (e.g. for run 16), certain plates showed a position effect i.e. the behaviour of cells depends on the where the well was located on the plate. In order to remove this effect, an additional mathematical approach can be applied. The data analysis files will clearly mention in case such an additional mathematical correction is applied and how it is carried out.

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[6] The value 3 is obtained following a hypothesis approach regarding two normal distributed variables, with different number of observations and unknown, but equal, variance. It thus takes into account the variance of the control and the chemicals.

[7] This simple test avoids that a single outlier is picked up as starting concentration of a significant effect.

6 Conclusion

Following the procedures explained within this report 4 separate runs have been performed, three with repeated exposure, 1 single exposure. Interpretation of the generated 4 sets of data is facilitated by this document as it contains all details of the data generation. At the same time the document allows HTS facilities to generate similar data in order to construct a more extensive data set.

The final conclusions of the data generated within the Seurat case study will be discussed in the final deliverable.

Annex 1: Overview of experiments

RUN	Cell treatment	Day of performance
Run 15	24h repeated exposure	04/05/2015
Run 16	24h repeated exposure	11/05/2015
Run 17	24h repeated exposure	03/08/2015
Run 18	24h single exposure	10/08/2015

Annex 2: Chemical list

Chemical name	CAS	Highest Conc (uM)	Chemical name	CAS	Highest Conc (uM)
1,1,1-Tris(hydroxymethyl)propan	77-99-6	250	Diphenylhydramine HCl	147-24-0	285
1,1,2,2-Tetrachloroethane	79-34-5	250	Dipropylene glycol	25265-71-8	250
1,2 Dichloroethane	107-06-2	250	Disulfiram	97-77-8	250
1,2-Dichloropropane	78-87-5	250	Dopamine HCl	62-31-7	310
1,4 Dichlorobenzene	106-46-7	250	Doxorubicin HCl	25316-40-9	106
2,3-Dimethylaniline	87-59-2	250	Eserine	57-47-6	250
2,5-Furantoine	108-31-6	250	Ethylene glycol	107-21-1	250
2-Ethylhexanoic acid	149-57-5	250	Ethylene glycol butyl ether	111-76-2	250
2-Methyl butyric acid	116-53-0	250	Fenofibrate	49562-28-9	30
2-Methyl-2-butanol	75-85-4	250	Furfural	98-01-1	250
3–acetamidophenol	621-42-1	250	Gliclazide	21187-98-4	250
5.5-diphenylhydantoin	57-41-0	125	Hydroquinpone	123-31-9	250
Acephate	30560-19-1	250	Isoniazid	54-85-3	250
Acetaminophen	103-90-2	250	Ketotifen fumarate	34580-14-8	340
Aflatoxin	1162-65-8	33	Methacrylonitrile	126-98-7	250
Amiloride HCl	2016-88-8	290	Methimazole	60-56-0	250
Amiodarone HCl	19774-82-4	26.5	Methotrexate	59-05-2	250
Amodiaquine 2HCl H2O	6398-98-7	13	Methyl viologen 2HCl H2O	1910-42-5	5
Ascorbate Na	134-03-2	11	Methylene bis(thiokyanate)	6317-18-6	250
Azathioprine	446-86-6	85	Methyleugenol	93-15-2	250
Bambuterol HCl	81732-46-9	110	Niacin	59-67-6	250
Betaine	107-43-7	10	Nimesulide	51803-78-2	250
Biotin	58-85-5	250	Octanoic acid	124-07-2	250
Bisacodyl	603-50-9	250	Pargyline	555-57-7	250
Bosentan	147536-97-8	100	Pentachlorobenzene	608-93-5	42
Buspirone HCl	33386-08-2	110	Pentachlorophenol	87-86-5	250
Butralin	33629-47-9	250	Pinacidil H2O	85371-64-8	268
Cadmium chloride	10108-64-2	250	Pioglitazone HCl	112529-15-4	110
Caffeine	58-08-2	100	Piperonyl butoxide	51-03-6	250
Carbamazepine	298-46-4	250	Piroxicam	36322-90-4	250
Carbidopa	28860-95-9	25	Propranolol HCl	839971	285
Chloramphenocil	56-75-7	250	Pyridoxine	65-23-6	250
Chlorpromazine HCl	69-09-0	93	Rifampicin	13292-46-1	100
Colchicine	64-86-8	250	Rotenone	83-79-4	35
Coumarin	91-64-5	250	Sorbitol	50-70-4	250
Cromolyn Na	15826-37-6	10.5	Sulindac	38194-50-2	250
Cyclohexanone	108-94-1	250	Tamoxifen	10540-29-1	15
Dantrolene Na	14663-23-1	21.5	tert-Butylacetate	540-88-5	250
Dazomet	533-74-4	250	Tetrachlorobenzene	95-94-3	17
Dexamethasone	50-02-2	250	Tetracycline HCl	64-75-5	90
Dichlobenil	1194-65-6	83	trans-2-Pentenoic acid	13991-37-2	250
Dichloromethane	75-09-2	250	Trifluralin	1582-09-8	83
Diclofenac Na	15307-79-6	270	Valeric acid	109-52-4	250
Diethanolamine	111-42-2	250	Valproic acid	99-66-1	250
Diethylcarbamazine citrate	1642-54-2	485	Zomepirac Na	64092-48-4	110

Annex 3: HTS workflow – updated on 27/04/2015

There are two computers:

–   Small one: used for preparing serial dilutions in DMSO and cell medium

–   Big one: used to run the exposure experiment

Small computer
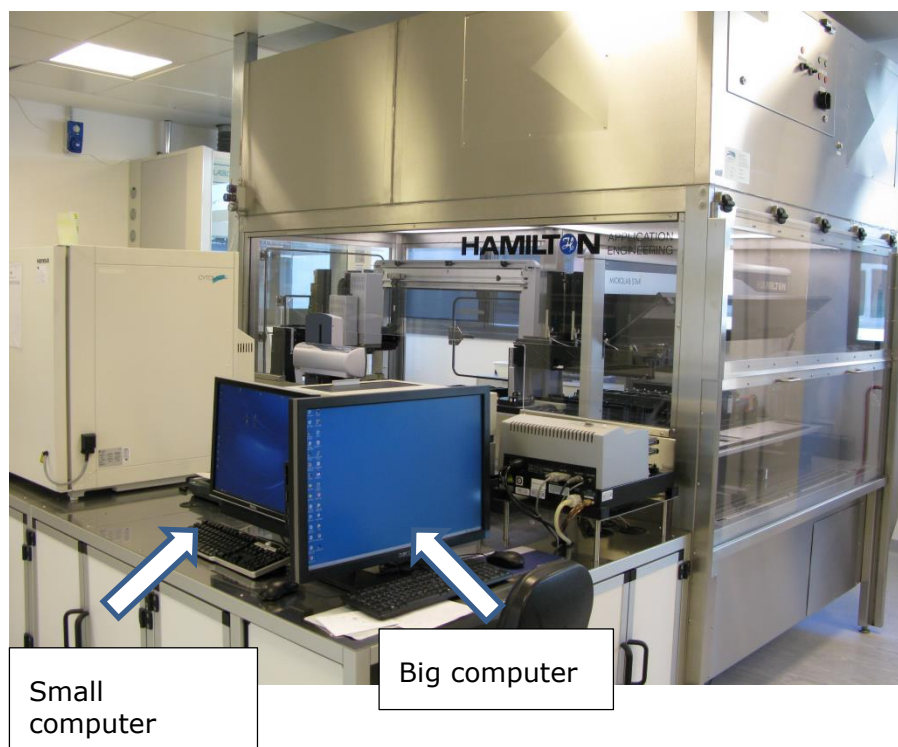
Big computer

<u>In case there is a black-out and there is need to restart the computers:</u>

1.   Switch on both computers
2.   The big computer will automatically launch a program to communicate with the small computer. Then there is the need to run another program for the communication with the small computer
3.   Still on big computer, click InetStar icon on desktop to open the program
4.   On the new window that just appeared, click "Hote" button
5.   Now the program is ready
6.   On the small computer, click QtHTSExecut icon on desktop to open the program
7.   Reduce the window just opened
8.   Still on small computer, click InetStarlet icon on desktop
9.   On the new window, click "Connecte" button
10.   Now the two computers are connected and HTS ready to be used

Note: never close the above windows opened in both computers

<u>Preparation of serial concentrations in DMSO and cell medium</u>

Loading the method

1.   On small computer, on desktop open the folder "qHTS-Starlet_SEURAT-1" containing the methods to prepare serial concentrations in DMSO and cell medium
2.   Open the sub-folder "Serial Dilutions_DMSO_1_1000"
3.   Double click on the method called "qHTS-Starlet Dilution:DPs_1000 (Hamilton Method)". The methods is to perform all steps needed to prepare chemical's serial dilutions in DMSO and cell medium and the control plate with positive and negative controls.

Fill the deep-well plates with cell medium

- HTS deck layout (as shown in Figure 2)

  - Put in the left-rack, tips of 1000 μL, 300 μL, 50 μL and 10 μL
  - Put the eleven deep-well plates and the control plate to be filled with cell medium
  - Put container with cell medium

Container 1	96-well plate holder	Deep well plate **C 1**	Deep well plate **C 6**	Deep well plate **C 7**	
Container 2	Tips Rack 1 (1 mL)	Deep well plate **C 2**		Deep well plate **C 8**	Tips Rack 1
Container 3	Tips Rack 2 (300 μL)	Deep well plate **C 3**		Deep well plate **C 9**	Tips Rack 2
Container 4	Tips Rack 3 (50 μL)	Deep well plate **C 4**	Deep well Control Plate	Deep well plate **C 10**	Tips Rack 3
Container 5	Tips Rack 4 (10 μL)	Deep well plate **C 5**	Container Cell Medium	Deep well plate **C 11**	Tips Rack 4

Figure 2

4. In the window just opened (in step 3), click on the icon of a "traffic light"
5. Then click the icon with a green horizontal arrow
6. Click yes on the message just shown
7. Click yes on the message just shown
8. Click yes on the message just shown to start filling the deep-well plates with cell medium.
9. After the cell medium is dispensed in all deep-well plates, the HTS method will proceed through the following steps of solutions' preparation.

Fill the daughter plates with DMSO

- HTS deck layout (as shown in Figure 3)

  - Remove the control plate to create space to place the daughter plates
  - Put three daughter plates
  - Put a new container with DMSO

Container 1	96-well plate holder	Deep well plate **C 1**	Deep well plate **C 6**	Deep well plate **C 7**	
Container 2	Tips Rack 1 (1 mL)	Deep well plate **C 2**	Daughter plate **(S1-S4)**	Deep well plate **C 8**	Tips Rack 1
Container 3	Tips Rack 2 (300 µL)	Deep well plate **C 3**	Daughter plate **(S5-S8)**	Deep well plate **C 9**	Tips Rack 2
Container 4	Tips Rack 3 (50 µL)	Deep well plate **C 4**	Daughter plate **(S9-S11)**	Deep well plate **C 10**	Tips Rack 3
Container 5	Tips Rack 4 (10 µL)	Deep well plate **C 5**	Container DMSO	Deep well plate **C 11**	Tips Rack 4

Figure 3

Prepare serial dilutions in DMSO

- HTS deck layout (as shown in Figure 4)
  - Remove container with DMSO
  - Put mother plate

Prepare serial dilutions in cell medium

No change of deck layout, just remove mother plate

Mixing of solutions in cell medium

Container 1	96-well plate holder	Deep well plate **C 1**	Deep well plate **C 6**	Deep well plate **C 7**	
Container 2	Tips Rack 1 (1 mL)	Deep well plate **C 2**	Daughter plate **(S1-S4)**	Deep well plate **C 8**	Tips Rack 1
Container 3	Tips Rack 2 (300 µL)	Deep well plate **C 3**	Daughter plate **(S5-S8)**	Deep well plate **C 9**	Tips Rack 2
Container 4	Tips Rack 3 (50 µL)	Deep well plate **C 4**	Daughter plate **(S9-S11)**	Deep well plate **C 10**	Tips Rack 3
Container 5	Tips Rack 4 (10 µL)	Deep well plate **C 5**	Mother plate	Deep well plate **C 11**	Tips Rack 4

Figure 4

No change of deck layout

Prepare control plate

- HTS deck layout (as shown in Figure 5)

    – Remove daughter plates
    – Put control mother plate
    – Put back the deep well control plate
    – Replace tips of 10 µL in the left rack

Container 1	96-well plate holder	Deep well plate **C 1**	Deep well plate **C 6**	Deep well plate **C 7**	
Container 2	Tips Rack 1 (1 mL)	Deep well plate **C 2**		Deep well plate **C 8**	Tips Rack 1
Container 3	Tips Rack 2 (300 µL)	Deep well plate **C 3**		Deep well plate **C 9**	Tips Rack 2
Container 4	Tips Rack 3 (50 µL)	Deep well plate **C 4**	Deep well Control Plate	Deep well plate **C 10**	Tips Rack 3
Container 5	Tips Rack 4 (10 µL)	Deep well plate **C 5**	Control mother plate	Deep well plate **C 11**	Tips Rack 4

Figure 5

Mixing of control plate

- HTS deck layout

    – Replace tips of 1000 µL in left rack

Preparation of deck for exposure experiment

- HTS deck layout (as shown in Figure 6)

The layout in Figure 6 is intended to replace the tips after every 6 h exposure in order to avoid cell medium dried within the tips as a mean to minimise cell detachment (in previous experiments tips were not replaced every 6 h exposure and cell medium dried was observed within the tips and this has potently contributed to cell detachment)

The practical solution is that after the first exposure is completed (let's say at 12), we soon replace manually the tips, in the left rack, with new ones. Then, the second exposure will be performed with new tips and as soon also this is completed (let 'say by 19) we replace again manually the tips in the left rack. In this way also the third exposure (at around midnight) is done with new tips.

For the fourth exposure (around 6 in morning), the tips in the right rack are used.

    – Change all tips and put in each tip-rack (left and right), tips of 300 µL

- Put a Lid-Holder
- Cover all deep-well plates (12) with a lid
- Change position of C1 deep well plate
- Put waste container

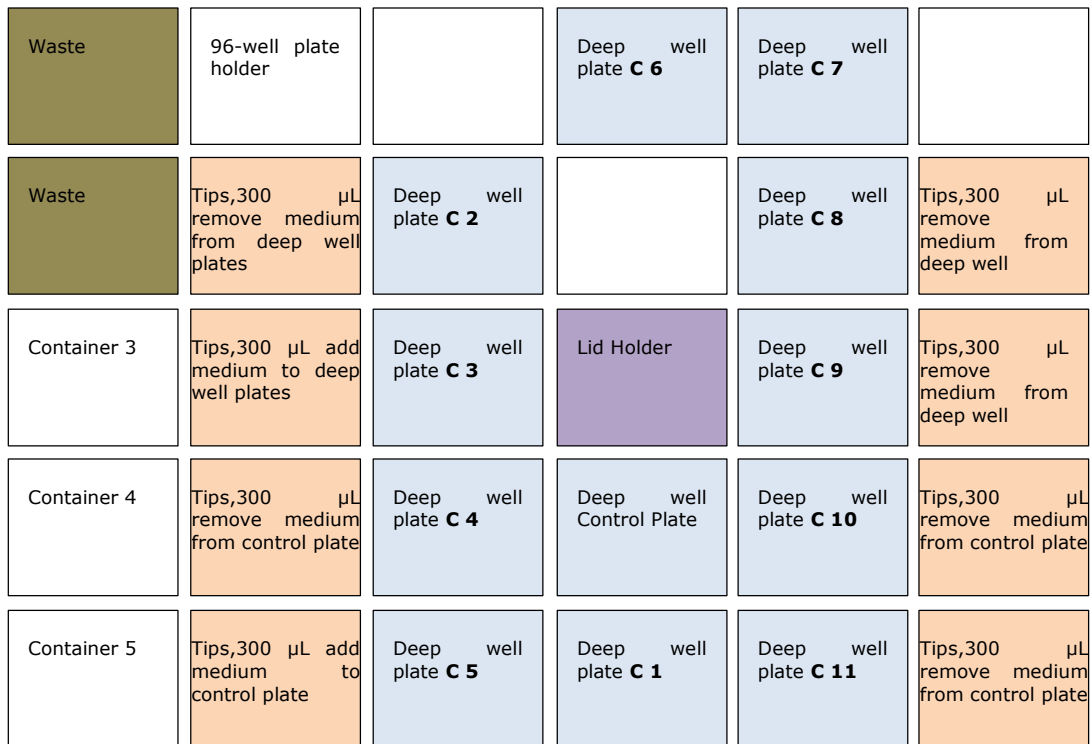|					
Waste	96-well plate holder		Deep well plate **C 6**	Deep well plate **C 7**	
Waste	Tips,300 µL remove medium from deep well plates	Deep well plate **C 2**		Deep well plate **C 8**	Tips,300 µL remove medium from deep well
Container 3	Tips,300 µL add medium to deep well plates	Deep well plate **C 3**	Lid Holder	Deep well plate **C 9**	Tips,300 µL remove medium from deep well
Container 4	Tips,300 µL remove medium from control plate	Deep well plate **C 4**	Deep well Control Plate	Deep well plate **C 10**	Tips,300 µL remove medium from control plate
Container 5	Tips,300 µL add medium to control plate	Deep well plate **C 5**	Deep well plate **C 1**	Deep well plate **C 11**	Tips,300 µL remove medium from control plate

Figure 6

10.   On small computer, close the method's windows

Start exposure experiment

1.   On big computer, open "My computer" folder
2.   Click on Start 1572
3.   Click on JRC folder
4.   Click on Input
5.   Open qHTS_SEURAT-1 folder. In here, there are previous excel file with the list of barcoded 96-well plates used for the previous exposure experiments.
6.   Open the most recent excel file (always with the directory IWL) to then modify the 96-well plate's barcodes. In the "Exposure" sheet, check that the number of test plates is 12 (if we only do one exposure at time). Save the excel with a new name indicating the date of the current experiment and use later on to start the exposure experiment.
Note: just to verify that the excel file was properly updated and saved, do right-click on the desktop to open Task Manager. Then click on Process to check if there is any excel file. If yes, click on the excel file and stop it.
7.   Put 96-well plates in the incubator and remember the rack number where you put them
8.   Still on big computer, on desktop open the ShortCut to Hamilton folder
9.   Open "Methods" folder
10.   Open qHTS-Star_SEURAT-1 folder
11.   Open Treatment Schedule Folder

12.   Click on SEURAT-1 Workflow (.wfl)
13.   In the window just opened, click on the icon of a "traffic light"
14.   Then click the icon with a green horizontal arrow
15.   Click ok on the message just shown
16.   Upload the excel file just previously prepared
17.   Close the excel file
18.   Click yes on the message just shown. According to the Rack number where you put the plates in incubator, update the column "volume"

Annex 4: Robotic arm instructions

PLATE ID ENTRY
1.   Switch on (green button) cellomics at least 15-20 min before using it. Check that the internal switch is also on (behind the front double doors of cellomics).
2.   Switch on the computer, log in (username: labadmin, password: LabAdmin65)
3.   Double click on "plate id" icon, enter username and password (cell, cell)
4.   Click on "add plate ID" and insert the invariable part of the barcode ID (eg TP10001, TP10002……TP100012 so you insert TP1000). Click "fill pattern" box → add the variable part of the ID (eg 01) in the designated box, (Eg. enter Start: 01 and End:12) → click on "add"→ click on "next".
5.   This step is only to use an existing protocol. For inserting a new protocol for the first time or if you have modified the name of an existing one, go to step 6.
Select the plates to be scanned (Hold "shift" key and select your plates) and then select the protocol from the "plate protocol box" (this is the protocol you have previously saved for plate scanning in "cellomics scan software"). Click on

 ↔ button. Click on "next" and click on "save changes". Go to step 9.

6.   This step is only to insert a complete new protocol or if you have modified the name of an existing one in the "cellomics scan software" of an already existing protocol.
Click on→"New plate protocol"→ Then, insert the name protocol name. Under "assay" select the folder where the protocol was saved → under "assay protocol" select your protocol→ under "form factor" select the appropriate plate. Finally click on "next".
7.   Select the area to be scanned & fields in the wells
8.   Click on "next" and click on "save changes". Go to step 5.
9.   Exit from wizard

CELLOMIC ROBOT ARM
10.   Go to robot arm
11.   Switch on the main power of robot arm
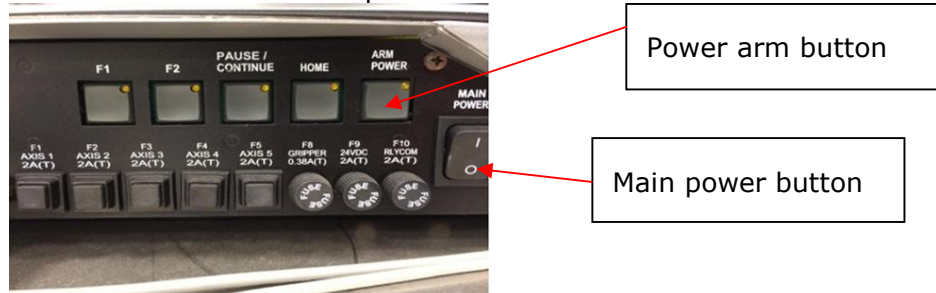
12.   Wait until robot stabilises (wait until "500D-CROS" is shown in the small screen near the robot arm main power button)
13.   Switch on "arm power" button and check that the red light is on (under the robotic arm). If it is not on, then the robot arm is not in the right position and should be placed on this position manually.
14.   Load the plates starting in column 1, from the top and keeping A1 well as it is indicated on the columns). Load plates keeping the same order you gave when filled the plate ID (Eg TP10001, TP10002……TP100012) SEURAT-1 from the highest to the lowest concentration being control plate is the last one. Don't forget to remove plate lid.

POLARA ARRAY
15.   Back to PC
16.   Double click on "polara array scan" icon
17.   Workspace→ change physical settings→ click on "yes"
18.   Click on "Teach" button → click on "unpark robot" → click on "yes" (the robot arm will be calibrated)

19.   Once the robotic arm has stopped, click on "park robot"→ click on "yes" and check that the red light under the robot arm is on.
20.   Click "exit" → close "physical settings " window (but do not close the "polara" software)
21.   On the left hand panel click on the "+" symbol besides arrayscan→ array scan plate ID → under the "method step" window/box select step 2"read microplate" → click on "properties" button → select "protocol" tab → click on "clear" button".
22.   On "plate ID" box insert, as before (step 4 and 14) , the invariable and variable part of the barcode as required. Tick on "use fill pattern"
23.   Click on "add" → click "ok" and close "method arrayscan plate ID" window
24.   At left hand panel, go to "plate ID" (on the left panel) → on the batch tab, ensure that the "array scan plate ID" is the method chosen (ensure that cellomics scan and cellomics view are closed). Insert the number of samples (if it has not been automatically done)
25.   Click on "schedule" and then on "run"
26.   Enter the password (cell) and click on "ok"
27.   Do not close/touch anything and go to "Polara Windows"
28.   Click on "start run"
29.   Wait until the "suspended bar" turns into yellow colour
30.   Click on "play" and then click on "start run"
31.   You can go to cellomics view to check that the image acquisition is correct

ROBOT SWITCH OFF

32.   Once all the plates have been scanned, press "Home" + "Continue" buttons together
33.   Release "Continue"
34.   Wait and release "Home"
35.   Switch off main power
36.   Switch off cellomics

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List of abbreviations and definitions

SOP	Standard Operating Procedure
PTP	Prototype Testing Platform
RTP	Routine Test Platform
HTS	High Throughput Screening
HCI	High Content Imaging
CT	Chemical Tube
TP	Test Plate
MP	Mother Plate
DP	Daughter/Deepwell Plate
ROS	Reactive Oxygen Species
DHE	Dihydroethidium
DMSO	Dimethyl sulfoxide
PBS	Phosphate Buffered Saline

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