The ToxTracker assay: a stem cell-based reporter assay for mechanistic carcinogenicity hazard screening

Validation report

Report prepared by:

Giel Hendriks[1,*], David Kirkland[2], Philippe Vanparys[3], Jan van Benthem[4] and Els Adriaens[5]

for the Organization for Economic Cooperation and Development Working Group of the National Coordinators of the Test Guidelines Program.

[1] Toxys, Oegstgeest, The Netherlands
[2] Kirkland Consulting, United Kingdom
[3] Consultant Genetic Toxicology, Belgium
[4] National Institute for Public Health and the Environment (RIVM), The Netherlands
[5] Adriaens Consulting, Aalter, Belgium

*Direct correspondence to:

Giel Hendriks, Ph.D.
Toxys
Leiden Bio Science Park
De Limes 7, 2342 DH, Oegstgeest
The Netherlands
+31 71 3322470
g.hendriks@toxys.com

Table of contents

1. List of abbreviations and acronyms

AOP	Adverse Outcome Pathway
BLR	Between-laboratory reproducibility
BMD	Benchmark Dose response
CA	Chromosomal Aberration assay
ECHA	European Chemicals Agency
EFSA	European Food Safety Authority
ECVAM	European Center for the Validation of Alternative Methods
GD	Guidance Document
GTTC	Genetic Toxicology Technical Committee at HESI
IATA	Integrated Approached to Testing and Assessment
ICH	International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use
IWGT	International Working group on Genetic Toxicology
IVIVE	*In Vitro* to *In Vivo* Extrapolation
LOAEL	Lowest observed adverse effect level
MLA	Mouse Lymphoma Assay
MN	Micronucleus Assay (*in vivo*)
MNvit	*in vitro* Micronucleus Assay
MoA	Mode of Action
NAM	New Approach Method
NOAEL	No observed adverse effect level
NRC	U.S. National Research Council
OECD	Organization for Economic Co-operation and Development
PARC	European Partnership for the Assessment of Risks from Chemicals
SCCS	Scientific Committee on Consumer Safety
SD	Standard deviation
SPSF	Standard Project Submission Form
TG	(OECD) Test Guideline
TGR	Transgenic rodent assay
TPF	Test Presubmission Form
VMT	Validation Management Team
WLR	Within-laboratory reproducibility
WNT	Working Group of National Coordinators of the OECD TGs program
WoE	Weight of Evidence

2. Introduction

1. In November 2016, an SPSF for a new test guideline (TG) for the ToxTracker assay was submitted to the OECD by The Netherlands. The SPSF application was primarily aimed at informing the OECD members on the ToxTracker assay and its applications for mechanistic genotoxicity and cancer hazard/risk assessment. The proposal was to perform an extensive prospective inter-laboratory validation study of the ToxTracker assay that should eventually lead to an official ToxTracker TG. The interlaboratory validation was performed according to OECD Guidance Document 34 (GD 34; OECD, 2005). On April 2017, the WNT accepted the SPSF on ToxTracker.

2. In parallel to the OECD submission, a Test Presubmission Form (TPF) for ToxTracker was submitted to the European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) on November 2016 (TM2016-03). In the TPF, ToxTracker was proposed as a novel approach for *in vitro* carcinogenicity hazard identification. The ToxTracker presubmission was reviewed by PARERE on May 2017. The preliminary response from PARERE on the presubmission was that ToxTracker would be a valuable expansion of the *in vitro* genotoxicity testing battery for regulatory applications. ToxTracker should not be considered as an alternative to the already existing *in vitro* genotoxicity assays, but as an additional tool that provides genotoxic mode-of-action information that would be useful to move towards the new paradigm of a mechanistically based risk assessment.

3. Genotoxicity testing is an essential part of chemical safety assessment. Recommendations for *in vitro* and *in vivo* testing are provided by various international guidelines, including for human pharmaceuticals in ICH S2(R1) (1), for industrial chemicals from ECHA (2), for foods and food additives from EFSA (3) and for cosmetic ingredients from SCCS (4). With the report from the U.S. National Research Council (NRC) in 2018 on Toxicity Testing in the 21st Century (5) as well as from various international initiatives to improve the safety testing approaches (i.e. US Tox21, EuToxRisk, PARC) comes a strong call for a major paradigm shift in toxicity testing. The use of novel approaches should allow chemical risk assessment to move beyond the classical endpoints and non-relevant test systems and extend the integration of mechanism-based toxicity test strategies that includes quantitative assessment of the mode-of-action (MoA) for (geno)toxic chemicals. In fact, in various legislations, there is already room for the use of New Approach Methods (NAMs) for chemical safety assessment. Data from these NAMs, for instance about the Mode-of-Action of genotoxic compounds, can be used in a Weight-of-Evidence (WoE) approach (4,6). The ToxTracker assay nicely fits into such an approach, as it provides mechanistic insight into the genetic toxicity and cancer hazard of chemicals.

ToxTracker is a mouse stem cell-based reporter assay that can identify genotoxic compounds with high accuracy (7,8). By combining different fluorescent reporter genes, the assay is able to provide insight into the MoA of genotoxic substances. The mechanism-based genotoxicity information can be particularly useful in AOP and WoE approaches and can contribute to the development of an Integrated Approach to Testing and Assessment (IATA).

3. Goals of the interlaboratory validation project

4. Although the primary use of Tox Tracker will be to provide insight into the MoA of genotoxic substances, such information is not valuable unless it has been demonstrated that the test system can reliably discriminate between mutagenic/genotoxic DNA reactive and not-genotoxic substances. Therefore, the primary goal of this validation project described in this report was to determine if ToxTracker is able to correctly predict the genotoxic properties of compounds. A broad selection of well-established genotoxic and non-genotoxic compounds was tested in ToxTracker to establish the sensitivity and specificity of the assay. In this interlaboratory validation study, the transferability and reproducibility of the ToxTracker assay was also established. The genotoxicity predictions for the tested compounds were compared from various repeat tests within a laboratory and between laboratories to calculate the within-lab and between-lab reproducibility.

5. ToxTracker is considered as an expansion of the toolbox of *in vitro* genotoxicity test to provide insight into the MoA of genotoxic substances. The secondary goal of the validation project was to investigate the MoA information that is provided by ToxTracker and the relevance for the genotoxicity prediction of the tested compounds. Information about the MoA of genotoxic and non-genotoxic compounds was applied to discriminate between direct and indirect genotoxic compounds and to better understand the results from ToxTracker in relation to results from the current standard *in vitro* and *in vivo* genotoxicity assays.

4. Scientific basis for the test method

6. ToxTracker combines six fluorescent reporter genes that are specifically activated by different cellular signaling responses that are associated with genotoxicity and carcinogenicity. The biomarker genes that are applied in ToxTracker were selected from toxicogenomics studies in which mouse stem cells were exposed to forty different genotoxic and non-genotoxic carcinogens (9). By detecting the activation of these reporter genes following chemical exposure, ToxTracker can discriminate between the induction of DNA damage, oxidative stress and protein damage and provide insight into the MoA of

genotoxic substances in a single test (Figure 1) (10). An accurate genotoxicity prediction in ToxTracker, and relevant MoA information is based on the combined profile of all six fluorescent reporter genes.

7. In ToxTracker, genotoxicity is primarily predicted by the activation of either of the two independent fluorescent reporters Bscl2-GFP and Rtkn-GFP. The Bscl2-GFP reporter is activated upon the formation of bulky DNA adducts and subsequent inhibition of DNA replication which is a potent activator of the DNA damage response. These replication-blocking DNA lesions often lead to the formation of mutations. Activation of the Rtkn-GFP genotoxicity reporter is associated with induction of DNA double-strand breaks. Many clastogenic compounds cause DNA damage by direct interaction with the DNA and typically activate both the Bscl2-GFP and Rtkn-GFP ToxTracker reporters. For such DNA reactive compounds, which may pose a cancer risk even at very low doses, a linear approach is typically used for risk assessment (11). In contrast, there are also substances that are genotoxic without directly interacting with the DNA (indirect genotoxicity) (11). Tubulin poisons, which interfere with chromosome segregation during mitosis, are indirect genotoxins and exposure to such agents can lead to aneuploidy (12). Activation of the Rtkn-GFP reporter and arresting of cells in mitosis is often observed for aneugens (13). The genotoxicity of compounds is further confirmed in ToxTracker by the Btg2-GFP reporter. Btg2 is a component of the P53-dependent DNA damage response and is involved in regulation of the G1/S cell cycle checkpoint but is also induced by various other cellular stressors. Also compounds that cause high levels of oxidative stress in the cells can indirectly affect the DNA. Insufficient or faulty repair of oxidative DNA damage can lead to mutations or chromosomal aberrations. Induction of oxidative stress is detected in ToxTracker by the Srxn1-GFP and Blvrb-GFP reporters that are activated by the two major antioxidant pathways in the cell. Also, protein misfolding or damage can lead indirectly to DNA damage. Activation of the unfolded protein response following chemical exposure is a potent trigger of apoptosis, leading to DNA breaks and chromosome fragmentation. Activation of the unfolded protein response is detected in ToxTracker by the Ddit3-GFP reporter. For these types of indirect damage, compensatory mechanisms exist, such as the presence of endogenous antioxidants. These compensatory mechanisms might give rise to a non-linear dose response and therefore a threshold approach is considered appropriate for such indirect genotoxins (14). To accurately predict the genotoxicity of compounds, both direct and indirect genotoxic effects should be considered. For this reason, integration of all six reporter genes in ToxTracker is required for reliable chemical safety assessment as well as for providing mechanistic information.

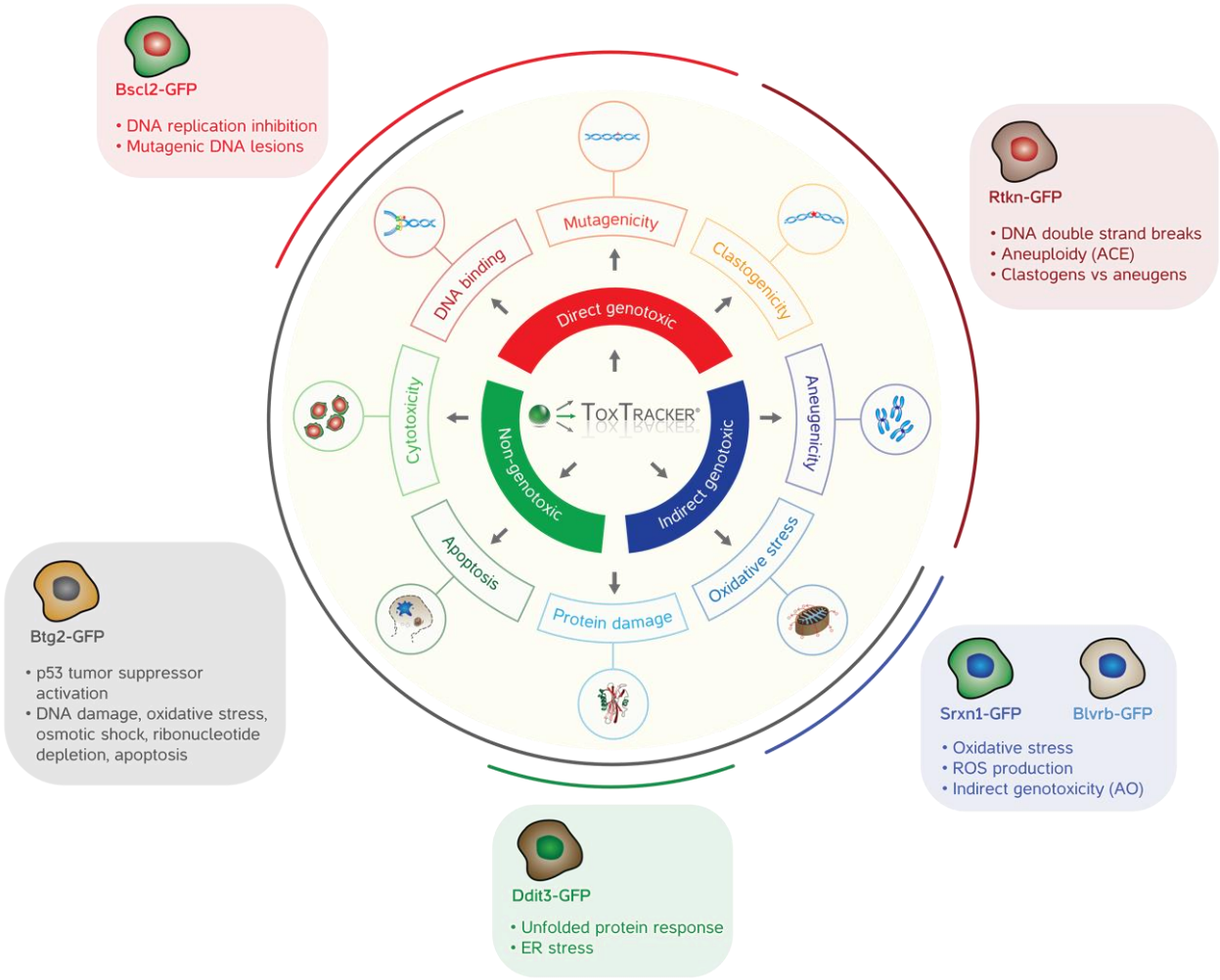


Figure 1: Schematic representation of the genotoxic and non-genotoxic endpoints covered in the ToxTracker assay.

8. ToxTracker is a GFP-based reporter assay, using untransformed mouse embryonic stem cells (mESC). Mutations in stem cells have been shown to play a crucial role in tumorigenesis. Stem cells are highly relevant for carcinogenicity hazard assessment and are highly suitable for mutation and genotoxicity testing due to their high cell proliferation rate (15). mESC are primary cells that are genetically stable, have an infinite life span and are proficient in all major DNA damage signaling and cell cycle regulation pathways. Activation of the fluorescent reporters in ToxTracker is measured by flow cytometry. GFP signals in individual cells are quantified in exposed and non-exposed cultures. Simultaneously, cytotoxicity of the tested compounds is determined by relative cell count.

5. The relationship between ToxTracker and *in vivo* biological effects

9. Extensive technical validation (>400 compounds) showed that ToxTracker combines a very high sensitivity (94%) and specificity (95%) for the detection of *in vivo* genotoxicity with the ability to provide insight into the MOA of genotoxic agents (7,8,13). ToxTracker contains reporters that predict induction of gene mutations and chromosomal damage with a high accuracy (>90%). In many cases, ToxTracker was able to correctly predict the genotoxic MoA of the tested compounds, including discrimination between direct and indirect genotoxicity, genotoxicity related to oxidative stress and differentiation between a clastogenic or aneugenic MoA. For the genotoxicity prediction, the differential activation of all six ToxTracker reporters was assessed. Activation of the ToxTracker reporter genes does show a strong correlation with the standard *in vitro* and *in vivo* genotoxicity assays. In a correlation study with 66 compounds from the ECVAM library of reference compounds (16), activation of the Bscl2-GFP reporter gene for induction of mutagenic DNA adducts showed a 93% correlation with a positive result in the Ames and/or MLA mutation assays. A negative result for the Bscl2-GFP ToxTracker reporter showed a 91% correlation with negative Ames and MLA results. Activation of the Rtkn-GFP reporter gene in ToxTracker that indicated the induction of DNA double strand breaks shows a very strong correlation of 92% with a positive result in the *in vivo* MN assay (17). A negative Rtkn-GFP reporter response correlated in 91% of the cases with a negative in vivo MN result. Interestingly, 30-40% of compounds that were negative in ToxTracker and negative in the *in vivo* MN assay did induce MN *in vitro*, underscoring the limited specificity of the *in vitro* MN assay (18). In many cases, the discrepancy between the *in vitro* MN and ToxTracker or *in vivo* MN assay could be explained by high levels of oxidative stress induction by the compound *in vitro*, e.g. tert-butylhydroquinone and resorcinol. Both compounds induce MN *in vitro*. ToxTracker was able to confirm the induction of oxidative stress and classified tert-butylhydroquinone and resorcinol as non-genotoxic, in line with the *in vivo* genotoxicity classification (Figure 2). Also for non-genotoxic compounds the mode-of-action information can be valuable. Induction of oxidative stress and the unfolded protein response have been shown to play a role chemical carcinogenesis (19,20).

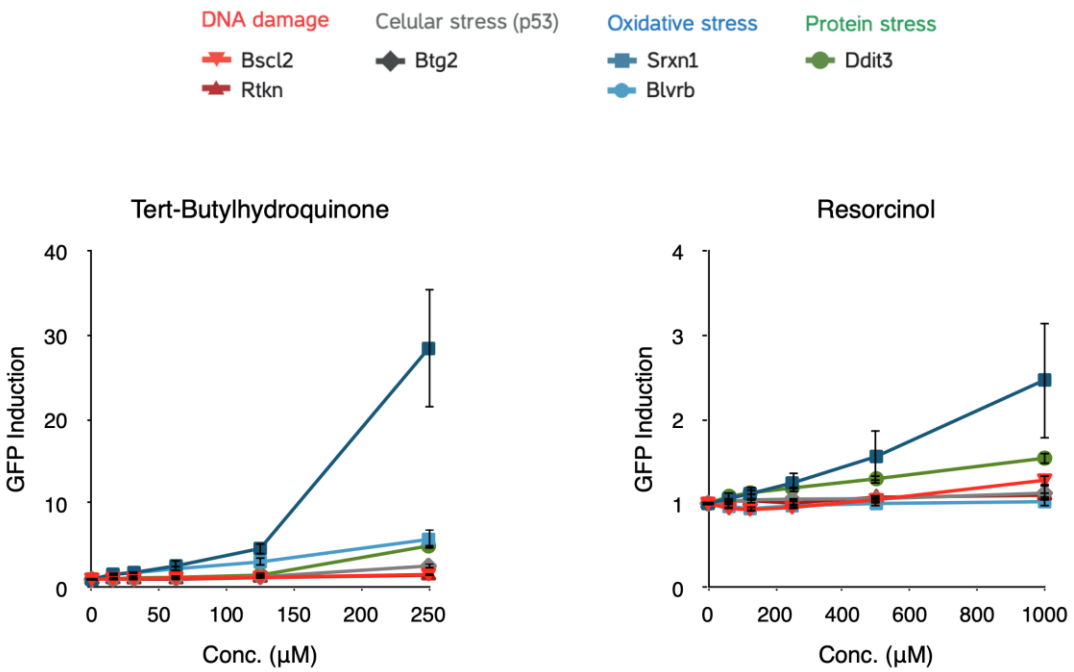


Figure 2: ToxTracker results for tert-butylhydroquinone and resorcinol. Both compounds activated the oxidative stress reporters (Srxn1-GFP; blue). None of the compounds activated the genotoxicity reporters (Bscl2-GFP/Rtkn-GFP; red)

6. Regulatory applications

10. The current standard battery of *in vitro* genotoxicity assays generally lacks the ability to provide information about the MoA of genotoxic substances. None of the existing OECD TG programs cover the same endpoints as ToxTracker. The battery of *in vitro* assays that are currently accepted for regulatory genetic toxicity testing include the bacterial Ames (TG471), mouse lymphoma (TG490) and mammalian cell (TG476) *in vitro* mutation tests. Chromosome damage is assessed by the *in vitro* micronucleus (TG487) and chromosome aberration (TG473) tests. This battery of *in vitro* genotoxicity assays generally has a sufficient sensitivity for genotoxicant identification but occasionally suffers from a relative high frequency of misleading positive test results (i.e., low specificity). The lack of mechanistic insight into these positive test results can be a serious challenge in the hazard assessment of chemicals and pharmaceuticals. By integrating the different reporter genes, ToxTracker can provide insight into the MoA of genotoxic compounds by predicting the induction of mutagenic DNA lesions, clastogenic effects, induction of aneugenicity and indirect genotoxicity caused by oxidative stress and protein damage in a single assay.

11. ToxTracker is already widely used by industry and research organizations for genotoxicity assessment (21–23). The ToxTracker assay is typically applied as

(i) a screening assay for early *in vitro* genotoxicity prediction or (ii) as a follow-up test to assess the MoA of compounds that give a positive or equivocal result in the regulatory accepted *in vitro* and *in vivo* genotoxicity assays. Application of ToxTracker as a screening assay can provide mechanistic information and indications on the compounds' genotoxicity that may indicate which classical *in vitro* genotoxicity tests would be most relevant to perform. Alternatively, ToxTracker would be recommended following a positive result from the current *in vitro* genotoxicity battery (Ames/MLA for mutagenicity and MNvit/CA for chromosome damage). ToxTracker is currently not intended to replace the existing TGs for *in vitro* genotoxicity testing. ToxTracker proved to be a valuable addition to the standard battery of *in vitro* genotoxicity assays for regulatory applications as it has the unique ability to reveal genotoxic modes of action and a number of non-genotoxic modes of action, such as oxidative stress and protein damage. ToxTracker results have already been included successfully in regulatory submissions to the FDA and US-EPA in the United States and to EMA, ECHA and EFSA in Europe as part of a WoE approach.

12. There is currently regulatory demand for more quantitative approaches for human risk assessment. In the standard battery of *in vitro* genotoxicity assays, often the no observed adverse effect level (NOAEL) or lowest observed adverse effect level (LOAEL) concentrations are calculated. However, the NOAEL/LOAEL can be adversely affected by study design and dose selection. As an alternative, quantitative dose-response modeling can be used to determine a robust potency metric such as the Benchmark Dose (BMD) (24). Application of the BMD for human risk assessment and calculation of health-based guidance values (HBGV) are already applied for regulatory genotoxicity testing (25–27). Data from the ToxTracker assay are highly suited for quantitative dose-response modeling using the BMD approach. By calculation the BMD for the different ToxTracker reporters, chemicals can be quantitatively ranked according to their potency to induce genotoxic through a specific MoA (26)(28). The BMD values from the ToxTracker reporters can be applied in IVIVE approaches (29), supporting the regulatory demands for quantitative assessment of human risk assessment.

7. Protocol for conducting the ToxTracker assay

13. The general protocol for conducting the ToxTracker assay has been published previously (8). See Annex 1 for the full ToxTracker protocol. The assays are performed in 96-well plates that contain the six different GFP reporter cell lines. The cells are seeded 24h prior to exposure to the test substances. Typically, five concentrations of a compound are tested in ToxTracker and four positive control compounds for each of the different GFP reporters are included in every test. Induction of the fluorescent ToxTracker reporters as well as the number of

viable cells is determined by flow cytometry after 24h exposure of the cells to the test compounds. Each compound is tested in three independent biological repeat experiments. The relative induction of the GFP reporters is calculated from the mean fluorescence from the three repeats by comparing treated cultures with the related vehicle control cultures. Cytotoxicity is determined based on relative cell count in exposed and control cultures.

14. Four positive control compounds have been selected to ensure the proper response of the different reporter genes. Compounds were selected based on their different MoA. Cisplatin is a DNA cross-linking agent that is included as a positive control for activation of the Bscl2-GFP, Rtkn-GFP and Btg2-GFP genotoxicity reporters. Aflatoxin B1 is a mutagenic substance that requires metabolic activation and is applied as positive control for S9 activity, Diethyl maleate activates the Nrf2-dependent antioxidant response and is applied as a positive control for the oxidative stress reporters Srxn1-GFP and Blvrb-GFP. Tunicamycin is a specific activator of the unfolded protein response and is the positive control for the protein damage reporter Ddit3-GFP. Each of the positive controls selectively activates the associated reporter genes, underscoring the specificity of the different reporters (Figure 3). The specificity of the reporters is not impacted by cytotoxicity, at least not up to the maximum acceptable cell survival level of 75%.

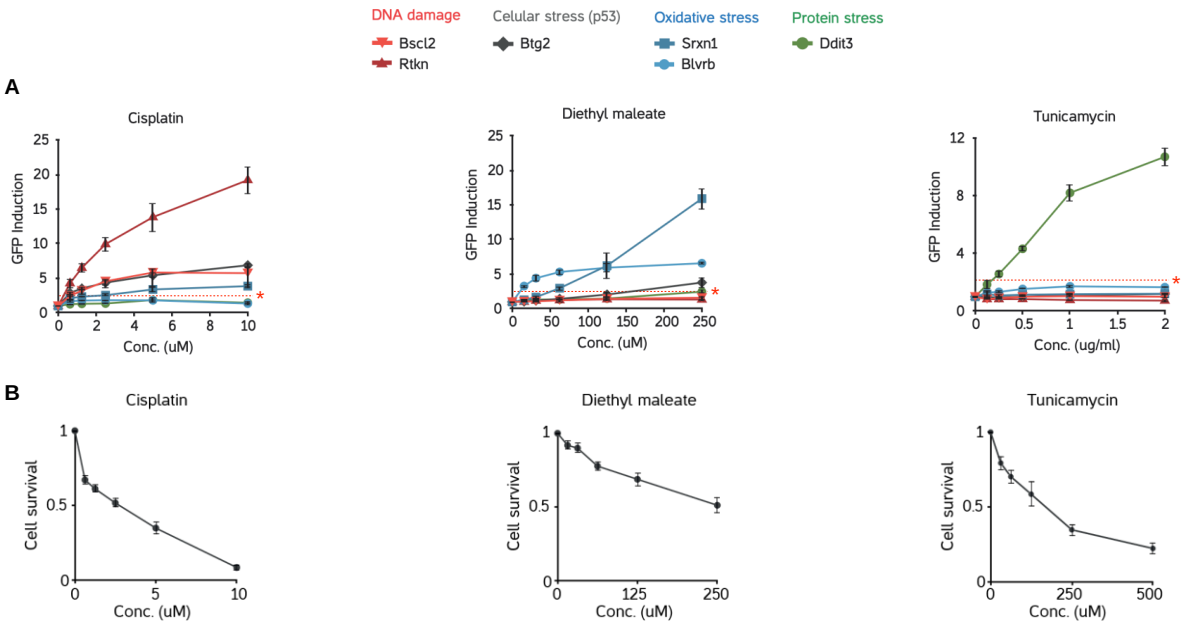


Figure 3: Selective activation of the ToxTracker reporter genes following exposure to the positive control compounds. A) The ToxTracker cells were exposed to increasing concentrations of the DNA damaging agent cisplatin, the oxidative stress-inducing agent diethyl maleate (DEM) or the unfolded protein response-activating compound tunicamycin. GFP induction levels in intact cells were determined by flow cytometry at 24 h after initiation of the exposure. B) Cell survival was determined by flow cytometry after 24-h exposure as the relative decrease in cell concentration compared with untreated controls. * 2-fold GFP reporter induction is the threshold for positive ToxTracker result.

15. Metabolization of compounds by the liver can have a major impact on their genotoxic properties. In common with the standard *in vitro* genotoxicity assays, ToxTracker uses S9 liver extract from Aroclor1254- or phenobarbital/β-naphthoflavone-induced rats for metabolic activation of compounds. The S9 metabolization protocol has previously been optimized for ToxTracker to allow for assessment of all the different endpoints that are covered by the assay. The ToxTracker assay is by default always performed in the absence and presence of a S9 metabolizing system.

16. The criteria for a positive or negative test result are defined in the ToxTracker protocol (Table 1). Only GFP reporter inductions at compound concentrations that induce <75% cytotoxicity are acceptable for ToxTracker analysis. A compound is classified as genotoxic if at least a 2-fold increase in expression of the Bscl2-GFP and/or Rtkn-GFP reporters is induced. Also, for the other four reporter genes, a 2-fold increase is GFP signals is applied as the threshold for a result to be considered positive. The 2-fold increase in GFP reporter activation as cut-off for a positive response is based on 3 times the standard deviation (SD) of the fluorescence levels in solvent control cell cultures. The validity of the 2-fold induction threshold for a positive ToxTracker result was

recently confirmed using a bootstrapping analysis of more than a 1000 solvent control ToxTracker cultures (28). For each ToxTracker reporter the distribution of background fluorescence levels was determined (30). Compounds are classified as non-genotoxic in ToxTracker if the induction levels of the genotoxicity reporter genes (Bscl2-GFP and Rtkn-GFP) is less than 1.5-fold. In the case that fluorescent reporter activation exceeds 1.5-fold but remains below a 2-fold increase, a weak positive score (+) is applied, but only on case of a clear dose response.

17. For assessment of the mode-of-action of compounds, also induction of the other ToxTracker reporters is included. Btg2 induction is associated with activation of the p53 tumor suppressor gene. Srxn1-GFP and Blvrb-GFP activation are indicative for the induction of cellular oxidative stress and Ddit3-GFP activation is associated with the unfolded protein response. For each independent ToxTracker experiment, activation of the different reporters is assessed, and compounds are classified accordingly. The criteria for a positive and negative test result are equal for all the ToxTracker reporters. For a final conclusion, the test results for every reporter gene from three independent repeat experiments are weighted according to the prediction model below (Table 2).

Table 1: Classification criteria for a positive ToxTracker test result and assessment calls for a single experiment.

GFP induction factor	Viability	Dose-response	Call
≥2.0 at 1 or more concentrations	≥0.25	Yes	+
≥2.0 at 1 or more concentrations	≥0.25	No	+
<1.5 at all concentrations	≥0.25 but approaches 0.25	No	-
<1.5 at all concentrations	≥0.25 but limited by precipitation	No	-
<1.5 at all concentrations	≥0.25 but with limited toxicity and not limited by precipitation	No	(-)
>1.5 but <2.0	≥0.25	Yes	(+)
>1.5 but <2.0	≥0.25	No	-

Table 2: Prediction model for ToxTracker.

Calls in 3 experiments (in any order)	Overall call for reporter with – or +S9 condition
+ + +	+
+ + (+)	+
+ + -	+
+ (+) (+)	+
- - -	-
- - (+)	-
- - +	-
- (+) (+)	-
(+) (+) (+)	E
+ (+) -	E
+ (+) (-)	E

18. To ensure a reliable classification of compounds, various quality controls and data acceptance criteria have been defined for the ToxTracker assay (see annex 1). Proper growth of the GFP reporter cell lines is monitored by tracking their proliferation rate. Criteria for the minimal proliferation rate of the cell lines is defined. In every ToxTracker experiment, positive controls for induction of DNA damage (cisplatin), S9 metabolization (aflatoxin B1), oxidative stress (diethyl maleate) and protein unfolding (tunicamycin) are included. Minimum induction levels for the different reporters have been defined. In the case that these minimum induction levels are not reached, an experiment should be discarded. Also, instructions for preparing compound dilutions and handling solubility issues are defined in the protocol.

8. Interlaboratory validation study management

19. Following the SPSF application by The Netherlands in 2016, an international interlaboratory ring trial for the ToxTracker assay was organized. The purpose of the trial was to establish the transferability and reproducibility of the assay. The goals of the interlaboratory trial were (i) to evaluate the accuracy of ToxTracker to predict *in vivo* genotoxicity and (ii) to validate the application of the mechanistic information that is provided by ToxTracker. Genotoxicity was defined as giving a positive *in vivo* result in the transgenic rodent (TgR), the PigA mutation, the MN, the CA and/or the Comet assays. A good intra- and inter-laboratory reproducibility is essential for any regulatory applications of the

assay. The accuracy to identify genotoxic compounds and identify their MoA will determine the impact and positioning of the assay in the standard strategy for genotoxicity testing.

20. The ToxTracker trial was organized according to OECD guidance document 34. The ring trial was organized by the validation management team (VMT) together with Toxys B.V. (The Netherlands). The VMT consisted of several recognized experts with established expertise in genetic toxicology and experience with interlaboratory validation studies for the OECD (Table 3). The VMT was responsible for defining the validation project structure, selection of the partner laboratories, setting the different milestones for the project and analysis of the test results. The VMT, excluding Toxys, was also responsible for the selection of compounds that would be included in the trial as well as for defining the test criteria and data acceptance. Toxys, as developer of the ToxTracker assay, was not involved in compound selection to prevent any selection bias or conflicts of interest.

Table 3: ToxTracker validation management team.

Validation Management Team	Affiliation	Country
David Kirkland	Kirkland consulting	UK
Philippe Vanparys	Gentoxicon	BE
Jan van Benthem	RIVM	NL
Els Adriaens	Adriaens consulting	BE
Giel Hendriks	Toxys	NL

21. The ToxTracker interlaboratory validation was performed by seven experienced laboratories from different industries (Table 4). All leading scientists in the ring trial are experienced in regulatory genotoxicity testing and many actively participate in scientific expert groups at the HESI GTTC and IWGT. The VMT regularly consulted with representatives from the participating laboratories about the progress of the ring trial. All important decisions about the validation study plan, study protocols, timelines, data analysis and acceptance were discussed and approved by the full ToxTracker validation team, including VMT and laboratories.

Table 4: ToxTracker ring trail laboratories

Partner	Industry	Country	Scientists involved
Pfizer	Pharma	US	Maik Schuler Maria Engel
Proctor & Gamble	Cosmetics	US	Stefan Pfuhler Ashley Allemang
GenenTech	Pharma	US	Tomomi Kiyota Jennifer Vogt Gabrielle Cole
Corteva agriscience	Agrochemicals	US	Raja Settivari Abby Myhre Stephanie Kellum
Roche	Pharma	CH	Andreas Zeller Valerie Naëssens
Labcorp	CRO	UK	Julie Clements Darren Kidd
Charles River Labs	CRO	CA	Annie Hamel Marise Roy Renato Cardoso

9. Compound selection

22. The selection of compounds for the interlaboratory validation trial was done by the VMT, excluding Toxys to prevent any conflict of interest or selection bias. The aim was to have a broad selection of compounds to cover as many chemical classes as reasonably possible with sufficient *in vitro* and *in vivo* genotoxicity data available. Compound selection was based on publicly available lists and databases (16,31,32). The procedure and considerations for selection of the compounds were similar as described for the JaCVAM international validation of the *in vivo* comet assay (33). The selected compounds can be divided into four groups: I) genotoxic carcinogens, II) genotoxic non-carcinogens, III) non-genotoxic carcinogens and IV) non-genotoxic non-carcinogens. The ToxTracker trial was solely focused on genotoxicity prediction and carcinogenicity was not considered as a criterion for compound selection. The compound list consists of organic and inorganic, aromatic and aliphatic molecules to cover a broad chemical space. Also, a number of compounds were selected that require metabolic activation in the liver. A full list of the selected compounds and their genotoxicity classification can be found in Annex 2. Genotoxicity of the compounds has been previously established and is based on the WoE from the various *in vitro* and *in vivo* mutation and genotoxicity assays that are publicly available. To have sufficient statistical power in the study, in total 64 chemicals were selected. Each

compound was tested independently in three laboratories. All compounds were coded and distributed exclusively by Els Adriaens. None of the participating laboratories were aware of the compound selection.

23. The ToxTracker interlaboratory validation project was divided into three phases. During the first phase, the ToxTracker assay was installed in the seven participating laboratories. The ToxTracker reporter cell lines, cell culture media and positive/negative control compounds were supplied by Toxys. Assay protocols were provided and an experienced scientist from Toxys visited each lab to set up the assay. During the lab training, proper culture of the reporter stem cell lines was ensured and the flow cytometer that was available at the laboratories was configured to run ToxTracker. A training set of 8 well-established genotoxic and non-genotoxic compounds was used to validate proper installation of the assay (Table 5). During a five-day training, each laboratory performed ToxTracker in three independent repeat experiments. The eight compounds were tested in the absence and presence of a rat liver extract (S9)-based metabolizing system. A template for ToxTracker data analysis (MS excel spreadsheet, see Annex 3) was shared by Toxys with the participating laboratories. An example data set from the installations in the laboratories can be found in Annex 4.

Table 5: Training set of compounds for ToxTracker installation

Compound	Cas number	Genotoxicity classification
Cisplatin	15663-27-1	Genotoxin, DNA cross-linker
Etoposide	33419-42-0	Genotoxin, Topo II-inhibitor
Aflatoxin B1	1162-65-8	Mutagen, requires S9 metabolization
Benzo[a]pyrene	50-32-8	Mutagen, requires S9 metabolization
Sodium arsenite	7784-46-5	Possible genotoxin, oxidative stress
Diethyl maleate	141-05-9	Non-genotoxin, oxidative stress
Tunicamycin	11089-65-9	Non-genotoxin, activation of UPR
Rosuvastatin	287714-41-4	Non-genotoxin

24. After the successful installation of ToxTracker, the participating labs were requested to run a limited proficiency test to show their ability to run the assay. For this, the VMT selected six compounds that were coded (Table 6). The laboratories received the blinded compounds and instructions for which solvent should be used. Each lab was requested to run a dose range finding

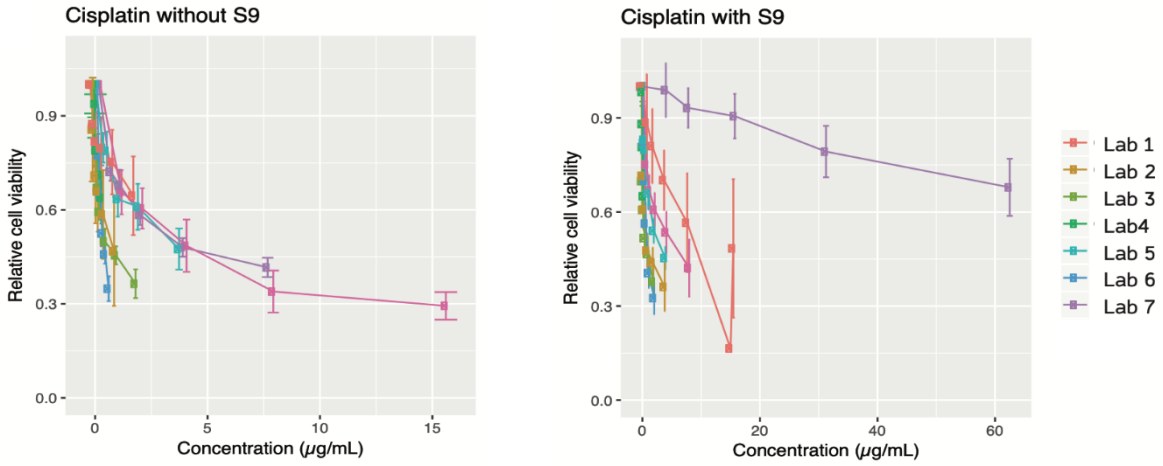
experiment to determine which concentrations to apply in ToxTracker. The top concentration for each compound should not exceed the 75% cytotoxicity induction threshold. Next, each compound was tested in the full ToxTracker protocol in the absence and presence of S9-mix.

Table 6: Compound selection for ToxTracker proficiency testing

Compound	Cas number	Genotoxicity classification
Cisplatin	15663-27-1	Genotoxin, DNA cross-linker
Ethyl methanesulphonate	62-50-0	Genotoxin, DNA methylation agent
Benzo[a]pyrene	50-32-8	Mutagen, requires S9 metabolization
Ampicillin	69-53-4	Non-genotoxin, antibiotic
D-mannitol	69-65-8	Non-genotoxin
o-anthranilic acid	118-92-3	Non-genotoxin

25. All test results from the second phase (proficiency testing) of the validation project were collected and analyzed by the VMT. First, the labs determined the cytotoxicity of the six compounds. Relative cell survival was determined after 24h exposure by relative cell count (Figure 4A). The labs selected the top concentration to apply in ToxTracker that induced 50-75% cytotoxicity, as instructed by the assay protocol (Figure 4B). In most of the cases, the compound concentrations that were selected by the different labs were in the same order of magnitude. Differences in dose selection could often be explained by either the approaches used for definition of cytotoxicity (i.e. differences in cell count by flow cytometers) and different criteria for solubility of compounds. Next, the labs performed the ToxTracker assay for each of the six reporter cell lines and indicated whether a positive response was observed (Figure 4C), according to the prediction model for ToxTracker (Table 1 and 2). All the laboratories were able to perform the ToxTracker assay according to the assay protocol. One lab reported issues with solubility of one of the compounds. No other significant issues were observed. The VMT collected the results and calculated the between-lab reproducibility for each of the toxicological endpoints that are included in ToxTracker (Figure 4D).

A



B **Maximum concentration tested (µg/mL)**

Laboratory	Without S9	With S9
Lab 1	1.9	15.6
Lab 2	0.97	3.9
Lab 3	1.9	1.9
Lab 4	0.24	0.24
Lab 5	3.9	3.9
Lab 6	0.48	1.9
Lab 7	7.81	62

C **Summary GFP induction**

Without S9	Bscl2	Rtkn	Srxn1	Blvrb	Ddit3	Btg2	With S9	Bscl2	Rtkn	Srxn1	Blvrb	Ddit3	Btg2
Lab 1	P	P	P	P	N	P	Lab 1	P	P	N	N	N	P
Lab 2	P	P	P	P	N	P	Lab 2	P	P	P	P	N	P
Lab 3	P	P	P	P	N	P	Lab 3	P	P	P	P	N	P
Lab 4	P	P	P	P	N	P	Lab 4	P	P	P	N	N	P
Lab 5	P	P	P	N	N	P	Lab 5	P	P	P	N	N	P
Lab 6	P	P	P	P	N	P	Lab 6	P	P	P	P	N	P
Lab 7	P	P	P	P	N	P	Lab 7	P	P	N	N	N	N

D

	Without S9					With S9				
Reporters	Genotoxicity	Oxidative stress	Prot.	p53	Reporters	Genotoxicity	Oxidative stress	Prot.	p53
BLR	100%	100%	100%	100%	BLR	100%	75%	100%	87.5%

Figure 4: Example of ToxTracker results from the validation laboratories. A) Cytotoxicity of the compound was determined by relative cell count after 24h exposure of the ToxTracker stem cells. B) The top concentration for ToxTracker was selected based on cytotoxicity of the compound. C) Classification of the compound for the different ToxTracker reporters. A positive result (P) was recorded in the case of at least a 2-fold increase of GFP expression. GFP induction levels lower than 2-fold resulted in a negative score (N), according to the criteria defined in tables 1 and 2. D) Between-lab reproducibility for the different toxicological endpoint that are investigated in ToxTracker.

26. Following the laboratory proficiency testing during the second phase of the ring trial project, the VMT concluded that all laboratories were able to perform ToxTracker according to the assay protocol. In this blinded study, all laboratories correctly identified ethyl methanesulphonate (EMS) and cisplatin (CIS) as genotoxic compounds (Table 8). Benzo[a]pyrene (BAP) when tested in the presence of S9 rat liver extract, was classified as a genotoxic compound in 6 of the 7 labs as expected. Lab 4 experienced solubility issues with BAP (likely related to prolonged storage) and therefore tested a lower concentration than the other labs which resulted in a non-genotoxic classification. Ampicillin, mannitol and anthranilic acid were correctly classified as non-genotoxic by all 7 labs. The overall between-lab reproducibility of ToxTracker in the proficiency test for the prediction of genotoxicity by the seven labs was 100% in absence of S9-mix and 97,9% in presence of S9-mix.

27. All six compounds were also analyzed for their genotoxic MoA (see Annex 5 for an overview of the results). CIS and EMS also induced the reporters for oxidative stress (Srxn1/Blvrb) and p53 activation (Btg2) but not the protein damage reporter (Ddit3). Together with the observed induction of both genotoxicity reporters (Bscl2/Rtkn), the ToxTracker results indicate that CIS and EMS are genotoxins that directly bind to the DNA. BAP activated all ToxTracker reporters indicating that the compound is a genotoxin with a broader MoA. The three tested non-genotoxic compounds did not induce any of the ToxTracker reporters.

Table 8: Genotoxicity classification of the phase 2 validation compounds.

	Bscl2 / Rtkn Without S9							Bscl2 / Rtkn With S9					
Laboratory	AMP	MAN	ANT	EMS	BaP	CIS	**Laboratory**	AMP	MAN	ANT	EMS	BaP	CIS
Lab 1	N	N	N	P	N	P	Lab 1	N	N	N	P	P	P
Lab 2	N	N	N	P	N	P	Lab 2	N	N	N	P	P	P
Lab 3	N	N	N	P	N	P	Lab 3	N	N	N	P	P	P
Lab 4	N	N	N	P	N	P	Lab 4	N	N	N	P	N	P
Lab 5	N	N	N	P	N	P	Lab 5	N	N	N	P	P	P
Lab 6	N	N	N	P	N	P	Lab 6	N	N	N	P	P	P
Lab 7	N	N	N	P	N	P	Lab 7	N	N	N	P	P	P
BLR (%)	100	100	100	100	100	100	**BLR (%)**	100	100	100	100	87.5	100
Overall (%)	**100**						**Overall (%)**	**97.9**					

N: Negative, GFP induction at all tested concentrations was <2 for the Bscl2 and Rtkn reporter cell lines.
P: Positive, GFP induction for at least one tested concenntration was ≥2 for the Bscl2 and/or Rtkn cell lines

28. After the ToxTracker installation and training of the laboratories, a number of modifications were made to the ToxTracker protocol by the VMT (See annex 1). Specific instructions were added about how to deal with poorly soluble compounds. Warming and sonication of poorly soluble compounds was added to the test protocol. In addition, specific instructions how to assess precipitation of the compounds was added. Precipitation of the test material should be addressed in the cell culture plates by microscopy at the end of exposure. Also, further instructions were added about quality controls for culture of the reporter cell lines, minimum induction levels of the GFP reporter cell lines for the positive control and criteria for a positive test result. Instructions for potentially required additional repeat experiments were added. Finally, a checklist for the scientists to make sure that all the crucial steps in the ToxTracker protocol were followed was added. The final ToxTracker validation protocol was approved by the VMT and all participating laboratories.

29. In the third phase of the ToxTracker ring trial, the seven laboratories received 24 or 30 coded compounds. Each compound was tested in three independent repeat experiments in the absence and presence of S9-mix. The laboratories received only instructions on which solvents to apply for preparing stock solutions. They therefore first needed to run a dose range finding experiment to determine the top concentration that should be tested in ToxTracker. The highest concentration should induce 50-75% cytotoxicity. In case of precipitation of the compound, the maximum soluble concentration would be applied. For non-cytotoxic compounds, the maximum concentration was set at 1 mg/ml. The mean GFP expression of the six ToxTracker reporters as well as cell concentrations was determined by flow cytometry and the results were

collected in a standard data analysis template (Annex 3). From this, the induction levels of the fluorescent reporters, as well as cytotoxicity of the compounds, were calculated. During the validation experiments, some technical support was provided by the VMT. Most of the questions were related to data acceptance and the requirement to perform a fourth repeat experiment. In two instances, a videoconference was organized with the VMT and all the participating laboratories to provide feedback and guidance on data acceptance.

30. The ToxTracker ring trial was performed between 2017 and 2022. Timelines for the validation project are summarized in Table 9. Assembly of the VMT and onboarding of the participating laboratories was completed in 2017. Technical training of the laboratories was completed in 2018. In 2018, all laboratories performed the second phase of the project, the proficiency testing. After approval of all phase 2 results by the VMT and establishment of the final validation protocol by the full ToxTracker consortium, the phase 3 validation was started in 2018. The timelines for completion of the experiments varied significantly between the laboratories. The Covid pandemic that started at the end of 2019 also had an impact on the performance of the validation experiments. When the labs completed their experiments, the test results were submitted to Els Adriaens for data analysis and compilation of the validation results. All test results were submitted in spring 2021 and analysis of the data was performed by the VMT. Compounds remained coded throughout the data analysis.

Table 9: Timeline of the interlaboratory validation ring trial of ToxTracker.

	Activity	Date	Status
1.	Assemble validation team	2017	✓
2.	Technical training of labs (phase 1)	2017-2018	✓
3.	Establish validation protocol	2018	✓
4.	Proficiency testing of labs (phase 2)	2018	✓
5.	Establish final ToxTracker protocol	2018	✓
6.	ToxTracker validation 24/30 compounds (phase 3)	2018-2021	✓
7.	Data analysis	2021	✓
8.	Draft validation report	2022	✓
9.	Review by OECD	2023	

10. Validation data analysis

31. Analysis of the ToxTracker validation trial results was performed by the VMT. All test results were compiled into a large database using the R programming language for statistical computing and graphics (34). Throughout the analysis, all compounds remained coded, except for the assay positive control compounds. During this analysis, the VMT first focused on data acceptance. The VMT first verified if the positive controls that were included in every experiment met the acceptance criteria, meaning did the control compounds induce the fluorescent reporters above the minimum threshold as set in the protocol at acceptable cytotoxicity levels (See protocol in Annex 2)? In some cases, one of the reporters did not meet all the acceptance criteria, e.g. cytotoxicity of the compounds was higher than the cut-off of 75% (Figure 3A) or activation of one of the genotoxicity reporters did not meet the minimal induction level (2-fold or 3-fold increase in GFP for the Bscl2-GFP or Rtkn-GFP respectively) (Figure 3B). In those cases, the VMT assessed if there was sufficient evidence that all cell lines were performing correctly, that the positive control compounds were active and if S9 metabolization was sufficient. In those cases, the VMT used their expert judgement to accept or reject the controls. In the data analysis database, these experiments were marked as “acceptable with restrictions”. In case the positive controls in a certain experiment did not meet the acceptance criteria, all the results from the test compounds in that experiment were discarded.

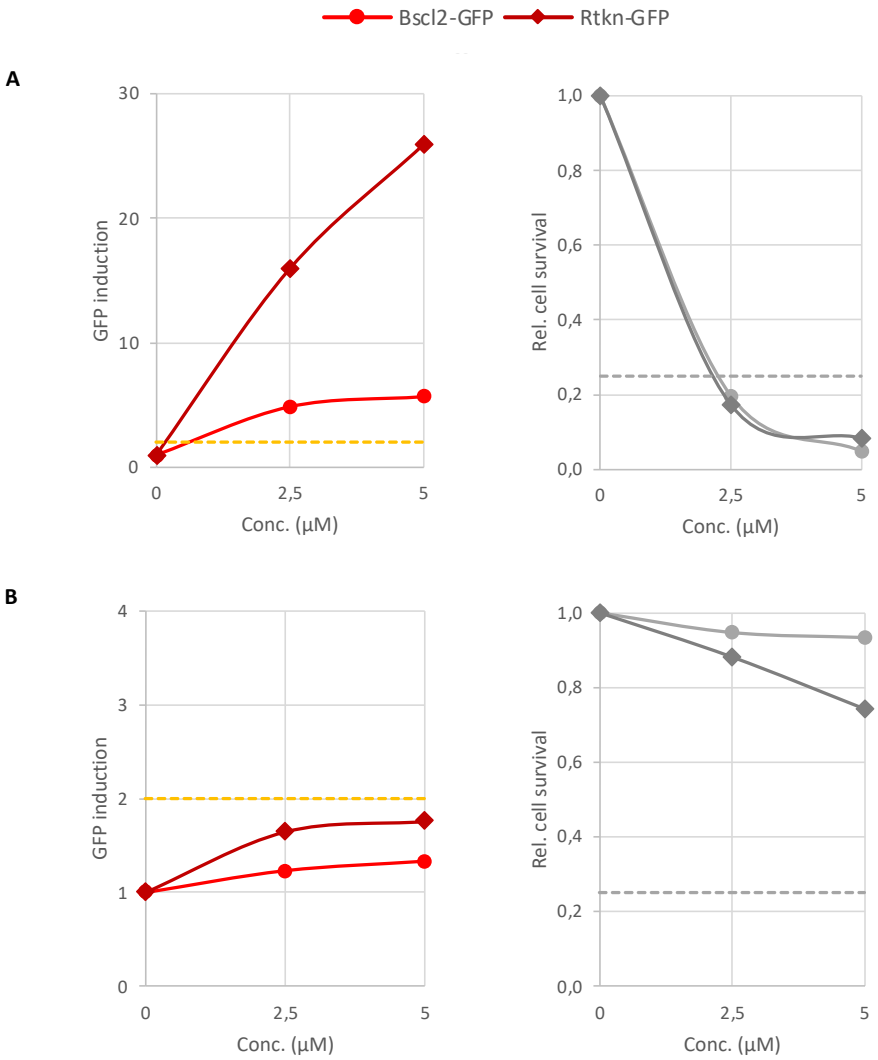


Figure 3: Examples of non-acceptable ToxTracker results. ToxTracker cells were exposed to the positive control compound cisplatin for 24h and activation of the Bscl2-GFP and Rtkn-GFP reporters as well as cytotoxicity was determined. **(A)** The ToxTracker reporters were activated above the 2-fold induction cut-off (left) but only at non-acceptable cytotoxicity levels (75%) (right). **(B)** Cytotoxicity levels were acceptable (right) but none of the ToxTracker reporters were activated above the 2-fold induction threshold.

32. From every participating laboratory, the ToxTracker results were analyzed for their acceptability according to the data acceptance criteria, with inclusion of expert judgment on occasions as discussed above. Inductions of the six fluorescent reporters following exposure to the test compounds as well as the relative cell survival was calculated. Figure 4 illustrates this for one of the test compounds. From each experiment, reporter activation was classified as positive, negative or inconclusive according to the ToxTracker prediction model (Table 1). Next, the classifications from the three independent repeat tests were weighted into an overall classification according to the prediction model (Table

2). Compounds were classified for their properties to induce genotoxicity (Bslc2/Rtkn), oxidative stress (Srxn1/Blvrb), protein damage (Ddit3) and p53 activation (Btg2). In case no clear overall classification could be made, compounds were classified as equivocal. If the results did not meet the data acceptance criteria, even after expert judgement, the compound was classified as inconclusive. For every compound, which was tested in each of three independent laboratories, the classifications were compared, and an overall classification was made based on a weighted calculation. After compiling the overview of the test results, the data analysis was approved by the VMT and the full validation consortium. After this approval, the compounds were decoded in May 2022.

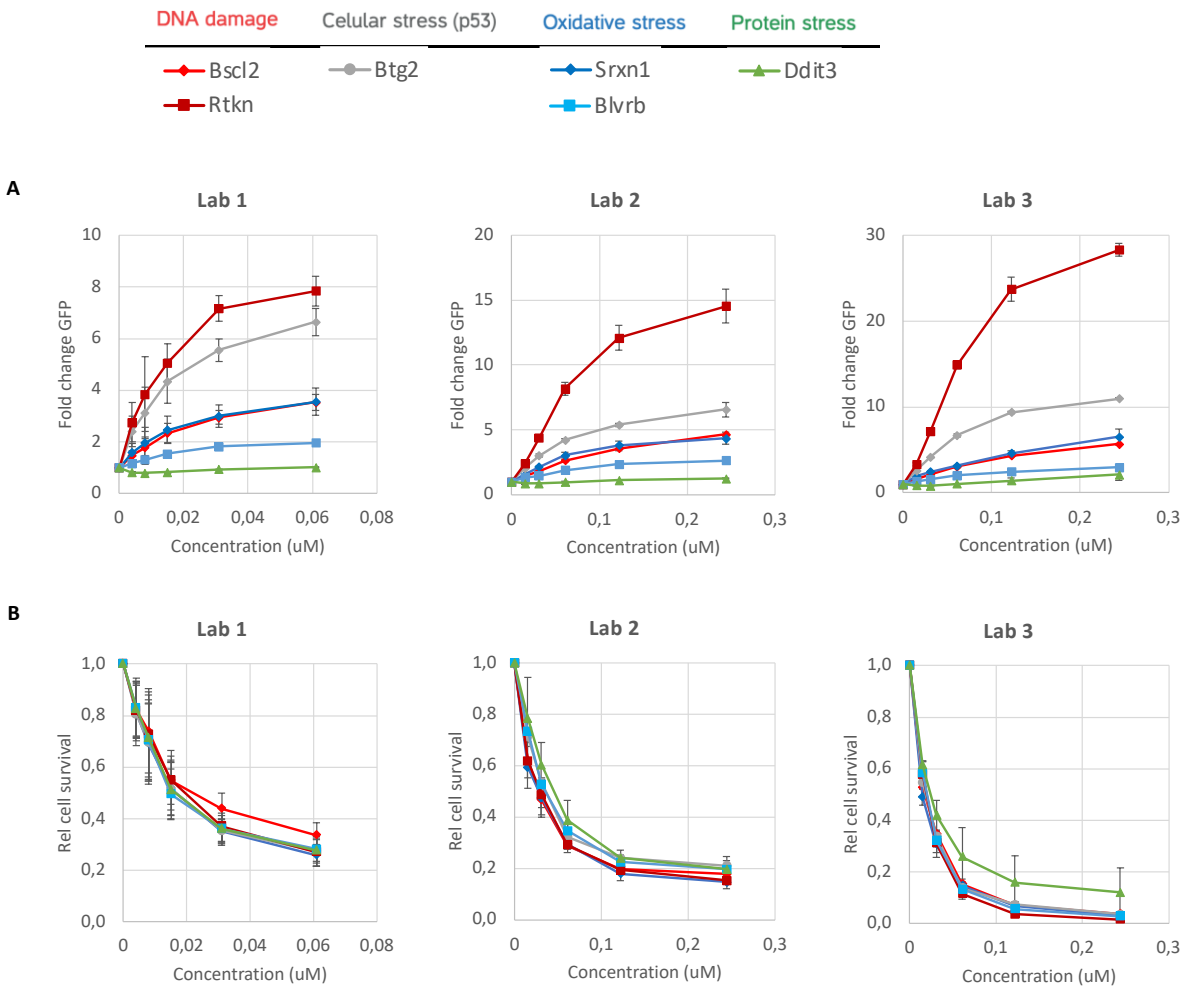


Figure 4: ToxTracker results for compound 1 from three independent laboratories. **(A)** Activation of the six different ToxTracker reporters following exposure to increasing concentration of the test compound. The Bscl2 and Rtkn reporters indicate genotoxicity, Srxn1 and Blvrb are induced by oxidative stress, Btg2 is associated with the p53 tumor suppressor response and Ddit3 is induced by protein misfolding. **(B)** Cytotoxicity of the compound is

determined by relative cell count in cultures exposed to the compound and their related vehicle control cultures.

33. First, the data from the seven validation laboratories were analyzed for their genotoxicity predictions. To this end, we first focused on the genotoxicity reporters (Bscl2/Rtkn) in ToxTracker and compared their responses to the expected genotoxicity classification of the 64 compounds that were included in this validation trial. The test compounds in this study were previously categorized as genotoxic or non-genotoxic by different expert committees and working groups based on the WoE from the standard battery of *in vitro* (Ames, MN, CA) and *in vivo* (TGR, MN) assays. Compounds were classified as genotoxic in ToxTracker when either one or both Bscl2-GFP and Rtkn-GFP reporters were induced above the 2-fold increase in GFP threshold. The Bscl2-GFP reporter indicated formation of bulky DNA adducts and the Rtkn-GFP reporter is activated upon formation of DNA double strand breaks. For 25 of the 32 expected genotoxic compounds, there was full concordance between the ToxTracker validation results and the genotoxicity classification from the standard genotoxicity testing battery. For three compounds (1,2-dimethylhydrazine, benzo[a]pyrene and 2,6-diaminotoluene), two labs reported a positive classification, but one lab classified the compounds as non-genotoxic. The negative result for 1,2-dimethylhydrazine in one of the labs was likely caused by a difference in concentration selection. The lab selected an 8-fold lower concentration to test in ToxTracker than the other two laboratories (31.5 µM instead if 250 µM) from the dose range finding experiment. The negative result for benzo[a]pyrene in one laboratory was probably related to inadequate metabolization by S9. There was also no cytotoxicity reported for the compound in presence of S9-mix, in contrast to the other laboratories. However, the positive control compound Aflatoxin B1 for S9 metabolization did result in the expected ToxTracker activation. Also 2,6-diaminotoluene was tested at a 15-fold lower concentration in one of the laboratories, likely causing the negative ToxTracker result (Table 10).

34. Four of the expected genotoxic compounds were classified as non-genotoxic in ToxTracker by all validation laboratories. Acrylonitrile was reported positive in the Ames mutation assay and showed mixed results in other *in vitro* genotoxicity assays (35). However, the *in vivo* MN and CA assays were negative for acrylonitrile and no DNA adduct could be detected following *in vivo* exposure. The carcinogenicity of acrylonitrile was suggested to be related to epigenetic mechanisms (36). Benzene is a very potent human carcinogen and *in vivo* mutagen (37,38). However, benzene is generally negative in the standard *in vitro* genotoxicity assays. Some benzene metabolites do induce MN or CA (39). Also, the oxidative stress and induction of oxidative DNA lesions has been reported. In general, *in vitro* metabolization by S9 does not support

the genotoxic effects of benzene. The lack of *in vitro* metabolization of benzene is the likely cause for the negative ToxTracker result. Cadmium chloride induces MN and CA *in vitro* and *in vivo* (40). Oxidative stress was reported to be an important mechanism for the genotoxicity of cadmium chloride. The ToxTracker validation laboratories classified cadmium chloride as non-genotoxic but did observe significant levels of oxidative stress. Finally, NDMA was classified as non-genotoxic whereas the nitrosamine compound is a very potent mutagen *in vivo*. *In vitro* metabolization of nitrosamines is poorly supported by S9 rat liver extract. NDMA was positive in the Ames mutation assay as well as the *in vitro* MN at concentration above 25 mM (41) In the ToxTracker validation, the maximum concentration that was tested by the laboratories was set at 1 mg/ml, thereby limiting the NDMA exposures to non-cytotoxic concentrations. NDMA has previously been classified as genotoxic in ToxTracker when tested up to 25 mM in the presence of hamster S9-mix.

Table 10: Genotoxicity classification, meaning activation of the Bscl2 or/and Rtkn genotoxicity reporters, of 32 *in vivo* genotoxic compounds in the ToxTracker trial.

											Final prediction		Weighted calculation	
	Compound		Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7		Overall		Pos	Neg
1	Etoposide	33419-42-0	P	P	P						P		1,00	
2	Mitomycin C	50-07-7	P		P	P					P		1,00	
3	Cisplatin	15663-27-1	P		P			P			P		1,00	
4	1,2- Dimethylhydrazine	306-37-6	P	P				N			P		0,67	0,33
5	1,2-dibromoethane	106-93-4				I	P	P			P		1,00	
6	Cyclophosphamide	6055-19-2	P		P	I					P		1,00	
7	2-Acetylaminofluorene	53-96-3				P	P		P		P		1,00	
8	Azidothymidine	30516-87-1				I	P		P		P		1,00	
9	ENU	759-73-9		P				P	P		P		1,00	
10	Acrylonitrile	107-13-1				N	N		N		N			1,00
11	Benzene	71-43-2		I	I		N				N			1,00
12	4,4′ -Oxydianiline	101-80-4		P				P	P		P		1,00	
13	Busulfan	55-98-1	P			P		P			P		1,00	
14	Ethyl methanesulfonate	62-50-0	P		I	P					P		1,00	
15	p-Chloroaniline	106-47-8				I	P	P			P		1,00	
16	7,12-Dimethyl-benzanthracene	57-97-6		P	P		I				P		1,00	
17	Benzo[a]pyrene	50-32-8		P	P		N				P		0,67	0,33
18	Cadmium Chloride	10108-64-2		N				N	N		N			1,00
19	Dimethylnitrosamine	62-75-9	N		I				N		N			1,00
20	2,4-Diaminotoluene	95-80-7				I	P	P			P		1,00	
21	o-Anisidine	90-04-0	P		I				P		P		1,00	
22	4-nitroquinoline-1-oxide	56-57-5			P			P	P		P		1,00	
23	6-Mercaptopurine	50-44-2	P		I	I					P		1,00	
24	Cytosine arabinose	147-94-4		P				P	P		P		1,00	
25	p-Phenylenediamine 2HCl	624-18-0	P				P	P			P		1,00	
26	8-Hydroxyquinoline	148-24-3			P	P		P			P		1,00	
27	9-Aminoacridine	90-45-9	P	P				P			P		1,00	
28	2,6-Diaminotoluene	823-40-5				N	P		P		P		0,67	0,33
29	3-Nitropropionic acid	504-88-1	P		P				P		P		1,00	
30	p-Anisidine	104-94-9		P	I				P		P		1,00	
31	5-fluorouracil	51-21-8	P	P		P					P		1,00	
32	Phenol	108-95-2	P				P	P			P		1,00	

35. From the 32 expected non-genotoxic compounds that were included in the validation trial, none were classified overall as genotoxic (Table 11), although for a number of compounds, a positive genotoxicity result was reporter by one laboratory.

Table 11: Genotoxicity classification, meaning activation of the Bscl2 or/and Rtkn genotoxicity reporters of 32 *in vivo* non-genotoxic compounds in the ToxTracker trial.

										Final prediction	Weighted calculation	
	Compound		Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Overall	Pos	Neg
33	Di(2-ethylhexyl)phthalate	117-81-7				I	N		N	N		1,00
34	Lead (ii) acetate	6080-56-4	P	N			I			E	0,50	0,50
35	2-Phenylphenol sodium salt	6152-33-6		N	I		I			N		1,00
36	Ropinirole hydrochloride	91374-20-8		I				N	N	N		1,00
37	Methyl carbamate	598-55-0		N				N	N	N		1,00
38	Cyclosporin A	59865-13-3	N		I	I				N		1,00
39	Sodium saccharin	128-44-9		N	N		N			N		1,00
40	Diethanolamine	111-42-2	N		N			N		N		1,00
41	Hexachloroethane	67-72-1			N			N	N	N		1,00
42	Melamine	108-78-1	P			I		N		E	0,50	0,50
43	Tunicamycin	11089-65-9	N					N	N	N		1,00
44	p-Nitrophenol	100-02-7		N				P	N	N	0,33	0,67
45	Phenanthrene	85-01-8	N	N	N					N		1,00
46	Tertiarybutylhydroquinone	1948-33-0	P			N		N		N	0,33	0,67
47	Benzyl alcohol	100-51-6		N	I		N			N		1,00
48	Vanilin	121-33-5	P		N				N	N	0,33	0,67
49	Erythromycin stearate	114-07-8	N					N	P	N	0,33	0,67
50	Sodium diclofenac	15307-79-6			I			P	N	N	0,50	0,50
51	o-anthranilic acid	118-92-3	N		N				N	N		1,00
52	Tolbutamide	64-77-7	N		N	I				N		1,00
53	2-ethyl-1,3-hexanediol	94-96-2	N	N	I					N		1,00
54	Chlorpheniramine maleate	113-92-8	N	N				N		N		1,00
55	Ampicillin trihydrate	7177-48-2				I	N		N	N		1,00
56	Sodium chloride	7647-14-5				N	N		N	N		1,00
57	D-mannitol	69-65-8	N		I	I				N		1,00
58	Allyl alcohol	107-18-6				I	N	N		N		1,00
59	(2-chloroethyl)trimethyl-NH3Cl	999-81-5				I	N	N		N		1,00
60	Sulfisoxazole	127-69-5				I	N	N		N		1,00
61	Sucrose	57-50-1				I	N		N	N		1,00
62	Cyclohexanone	108-94-1	N		N	I				N		1,00
63	1-Nitropropane	108-03-2		N	N		N			N		1,00
64	Phenformin HCl	834-28-6		N				N	N	N		1,00

In most cases (lead acetate, tert-butyl hydroquinone, vanillin, erythromycin stearate and diclofenac), the lab only observed activation of the Rtkn-GFP reporter which indicates the formation of DNA strand breaks but not the Bscl2-GFP reporter for the formation of bulky DNA lesions and DNA replication

inhibition. This pattern of reporter activation is typically observed for compounds that are indirect genotoxins, often secondary to the induction of oxidative stress. Indeed, for lead acetate and tert-butyl hydroquinone, indirect genotoxicity due to oxidative stress has been reported (42)(43). It is therefore interesting and relevant that, for all of these Rtkn-GFP reporter positive compounds, the laboratories reported activation of the Srxn1 and Blvrb reporters for oxidative stress. P-nitrophenol activated both Bscl2 and Rtkn genotoxicity reporters in one laboratory, indicating direct DNA reactivity, but this result could not be confirmed by the other laboratories. P-nitrophenol is negative in the standard battery of *in vitro* genotoxity assays, but there are various reports of positive CA and MN tests *in vivo* (16). Also, melamine, a non-genotoxic compound, was classified as genotoxic by one laboratory but was negative in the other two labs.

36. From the weighted calculations, the overall sensitivity and specificity of ToxTracker to identify (*in vivo*) genotoxic compounds was calculated. The calculations were done for 59 compounds for which acceptable data was collected from at least 2 labs. Compounds for which data was only available from 1 laboratory were excluded from the calculations. In this validation trial, the ToxTracker assay correctly identified genotoxic compounds with a sensitivity of 87% (26 of 30 expected positives) and a specificity of 90% (26 of 29 expected negatives). The accuracy of identifying genotoxic compounds in this validation study was very much in line with previous validation reports by Toxys (8).

11. Genotoxic mode-of-action assessment in ToxTracker

37. The second main objective of this interlaboratory validation study was to investigate the added value of the MoA information that is provided by ToxTracker for the genotoxicity prediction of compounds, and this was a meaningful objective since, as described above, it was shown that ToxTracker could reliably discriminate between genotoxic and non-genotoxic substances. ToxTracker is proposed as an expansion of the *in vitro* genotoxicity test battery to provide insight into the MoA of genotoxic compounds. Especially for compounds for which conflicting results have been reported from the various *in vitro* genotoxicity assays or between the *in vitro* and *in vivo* tests, insight into the MoA of compounds can help to explain the differences and to better classify compounds (13,44). The ToxTracker assay combines six different reporters to investigate the induction of DNA damage, oxidative stress and protein damage. Activation of the Bscl2 and/or Rtkn reporters indicate genotoxicity, Srxn1 and Blvrb activation shows induction of oxidative stress, Ddit3 is associated with protein unfolding and Btg3 activation is linked to the p53-associated cellular

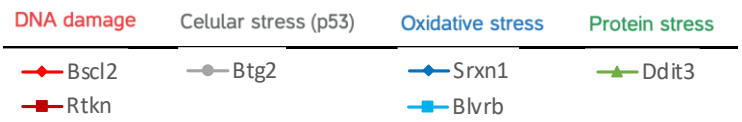
stress response. By assessing the differential induction of these reporters, the assay can provide insight into the MoA of genotoxic compounds. It is therefore essential to include the full panel of six ToxTracker reporters for genotoxicity prediction and MoA assessment. In the validation trial, induction of all six reporters was determined by the laboratories following exposure to the 32 genotoxic and 32 non-genotoxic compounds. A summary of the results is shown in Table 12. Every compound was tested in three laboratories. The results from the different labs for every reporter were combined into an overall classification using a weighted calculation. An overview of all the test results can be found in Annex 6.

Table 12: Summary of the combined ToxTracker reporter activations from the different validation laboratories in the ring trial.

Code	Compound	Cas#	Bscl2 -S9	Bscl2 +S9	Rtkn -S9	Rtkn +S9	Overall	Srxn1 -S9	Srxn1 +S9	Blvrb -S9	Blvrb +S9	Overall	Ddit3 -S9	Ddit3 +S9	Overall	Btg2 -S9	Btg2 +S9	Overall
1	Etoposide	33419-42-0	P	P	P	P	P	P	P	N	N	P	N	N	N	P	P	P
2	Mitomycin C	50-07-7	P	P	P	P	P	P	P	P	P	P	N	N	N	P	P	P
3	Cisplatin	15663-27-1	P	P	P	P	P	P	P	P	P	P	N	N	N	P	P	P
4	1,2- Dimethylhydrazine	306-37-6	P	P	P	P	P	P	P	P	P	P	N	N	N	P	P	P
5	1,2-dibromoethane	106-93-4	N	N	P	P	P	P	P	P	P	P	N	N	N	N	N	N
6	Cyclophosphamide	6055-19-2	N	P	N	P	P	N	P	N	P	P	N	N	N	N	P	P
7	2-Acetylaminofluorene	53-96-3	N	N	P	P	P	P	P	N	N	P	N	N	N	N	N	N
8	Azidothymidine	30516-87-1	P	P	P	P	P	P	P	P	P	P	N	N	N	P	P	P
9	ENU	759-73-9	P	P	P	P	P	P	P	P	P	P	N	N	N	P	P	P
10	Acrylonitrile	107-13-1	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
11	Benzene	71-43-2	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
12	4,4' -Oxydianiline	101-80-4	P	P	P	P	P	P	P	P	P	P	N	N	N	P	P	P
13	Busulfan	55-98-1	P	P	P	P	P	P	P	P	P	P	N	N	N	P	P	P
14	Ethyl methanesulfonate	62-50-0	P	P	P	P	P	P	P	P	P	P	N	N	N	P	P	P
15	p-Chloroaniline	106-47-8	N	N	P	P	P	N	P	N	P	P	N	N	N	N	P	P
16	7,12-Dimethyl-benzanthracene	57-97-6	N	N	N	P	P	N	N	N	P	P	N	N	P	N	P	P
17	Benzo[a]pyrene	50-32-8	N	P	N	P	P	N	P	N	P	P	N	P	P	N	P	P
18	Cadmium Chloride	10108-64-2	N	N	N	N	N	P	P	P	P	P	P	P	P	N	N	P
19	Dimethylnitrosamine	62-75-9	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
20	2,4-Diaminotoluene	95-80-7	P	P	P	P	P	P	N	P	P	P	P	P	P	P	P	P
21	o-Anisidine	90-04-0	N	N	P	P	P	P	P	P	P	P	N	N	N	P	P	P
22	4-nitroquinoline-1-oxide	56-57-5	P	P	P	P	P	P	N	P	N	P	N	N	N	P	P	P
23	6-Mercaptopurine	50-44-2	N	N	P	P	P	P	P	N	N	P	N	N	N	P	P	P
24	Cytosine arabinose	147-94-4	P	P	P	P	P	N	N	P	P	P	N	N	N	P	P	P
25	p-Phenylenediamine 2HCl	624-18-0	P	P	P	P	P	P	P	P	P	P	N	N	N	P	P	P
26	8-Hydroxyquinoline	148-24-3	N	N	P	P	P	P	P	N	P	P	P	P	P	P	P	P
27	9-Aminoacridine	90-45-9	N	N	P	P	P	P	P	N	N	P	N	N	N	P	P	P
28	2,6-Diaminotoluene	823-40-5	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P
29	3-Nitropropionic acid	504-88-1	P	P	P	P	P	P	P	P	P	P	N	N	N	P	P	P
30	p-Anisidine	104-94-9	P	P	P	P	P	P	P	P	P	P	P	N	P	P	P	P
31	5-fluorouracil	51-21-8	P	P	P	P	P	P	P	P	P	P	N	N	N	P	P	P
32	Phenol	108-95-2	N	N	P	P	P	P	P	P	P	P	N	N	N	P	P	P
33	Di(2-ethylhexyl)phthalate	117-81-7	N	N	N	N	N	N	N	N	N	N	P	N	P	N	N	N
34	Lead acetate	6080-56-4	N	N	N	N	N	P	P	P	P	P	N	N	N	N	N	N
35	2-Phenylphenol	6152-33-6	N	N	N	N	N	P	P	N	P	P	P	P	P	N	N	P
36	Ropinirole hydrochloride	91374-20-8	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
37	Methyl carbamate	598-55-0	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
38	Cyclosporin A	59865-13-3	N	N	N	N	N	P	P	P	P	P	P	P	P	N	N	N
39	Sodium saccharin	128-44-9	N	N	N	N	N	P	P	P	P	P	N	N	N	N	N	N
40	Diethanolamine	111-42-2	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
41	Hexachloroethane	67-72-1	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
42	Melamine	108-78-1	N	N	N	N	N	N	N	N	N	P	N	N	N	N	N	N
43	Tunicamycin	11089-65-9	N	N	N	N	N	N	N	N	N	N	P	P	P	N	N	N
44	p-Nitrophenol	100-02-7	N	N	N	N	N	N	N	N	N	N	P	P	P	N	N	N
45	Phenanthrene	85-01-8	N	N	N	N	N	P	P	P	P	P	P	P	P	N	N	N
46	Tertiarybutylhydroquinone	1948-33-0	N	N	N	N	N	P	P	P	P	P	P	P	P	P	P	P
47	Benzyl alcohol	100-51-6	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
48	Vanilin	121-33-5	N	N	N	N	N	P	P	N	P	P	N	N	N	N	N	P
49	Erythromycin	114-07-8	N	N	N	N	N	P	P	N	N	P	P	N	P	N	N	N
50	Sodium diclofenac	15307-79-6	N	N	N	N	N	P	P	N	N	P	P	P	P	P	E	P
51	o-anthranilic acid	118-92-3	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
52	Tolbutamide	64-77-7	N	N	N	N	N	P	P	N	N	P	P	P	P	N	N	N
53	2-ethyl-1,3-hexanediol	94-96-2	N	N	N	N	N	P	P	N	N	P	N	N	N	N	N	N
54	Chlorpheniramine maleate	113-92-8	N	N	N	N	N	P	P	P	P	P	N	N	N	N	N	N
55	Ampicillin trihydrate	7177-48-2	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
56	Sodium chloride	7647-14-5	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
57	D-mannitol	69-65-8	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
58	Allyl alcohol	107-18-6	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
59	Chlormequat chloride	999-81-5	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
60	Sulfisoxazole	127-69-5	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
61	Sucrose	57-50-1	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
62	Cyclohexanone	108-94-1	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
63	1-Nitropropane	108-03-2	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
64	Phenformin HCl	834-28-6	N	N	N	N	N	N	N	N	N	N	P	P	P	N	N	N

38. In general, for the genotoxic compounds with an expected direct DNA damaging MoA, both Bscl2 and Rtkn genotoxicity reporters were activated, e.g.

etoposide, mitomycin C, cisplatin, 5-fluorouracil. Activation of the Bscl2 reporter indicates the formation of DNA adducts that inhibit DNA replication. Bscl2 activation generally correlates with a positive result in the standard *in vitro* bacterial (Ames) and mammalian cell gene mutation assays (MLA/HPRT) and indicates a mutagenic MoA. The Rtkn reporter is activated upon the formation of DNA double strand breaks and indicates a clastogenic MoA. Rtkn activation correlates strongly with a positive response in the *in vitro* and *in vivo* MN and CA clastogenicity assays. Compounds that require metabolization in the liver activated the genotoxicity reporters only in the presence of S9-mix, e.g. cyclophosphamide, benzo[a]pyrene and 7,12-Dimethyl-benzanthracene. For most of the genotoxic compounds, activation of the p53 tumor suppressor-associated Btg2 reporter and occasionally the oxidative stress reporters Srxn1 and Blvrb was also observed. However, from the dose response graphs for the genotoxic compounds, induction of the genotoxicity reporters is clearly the primary response (example for etoposide shown in Figure 3). For a number of compounds, only activation of the Rtkn, but not the Bscl2 genotoxicity reporter was observed. This is often observed for compounds causing indirect genotoxic effects, including aneugens or oxidative stress-inducing compounds. For example, the major mechanistic pathway for the genotoxicity of 1,2-dibromoethane is through binding to the cellular antioxidant GSH (45). Also, for 8-hydroquinoline, the primary genotoxic MoA was reported to occur through induction of oxidative stress (46). Accordingly, 1,2-dibromoethane and 8-hydroquinoline primarily activated the Srxn1 oxidative stress reporter as well as the Rtkn reporter for clastogenic DNA lesions, suggesting that genotoxicity (clastogenicity) of these compounds is caused by oxidative stress (Figure 5).



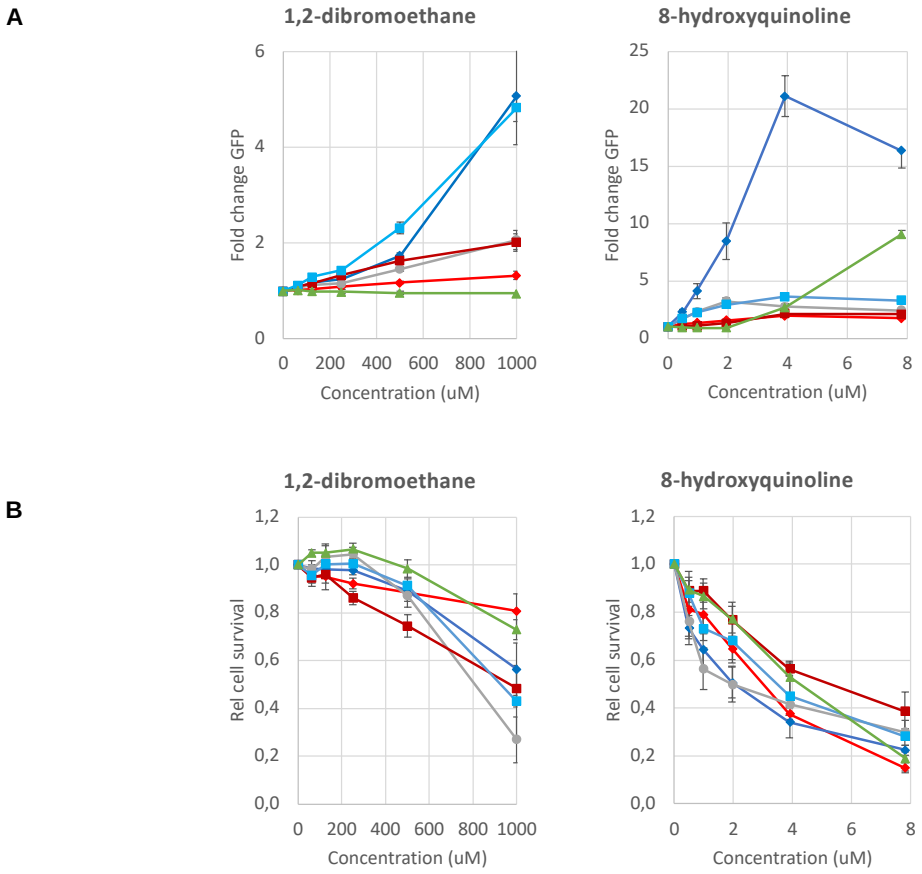
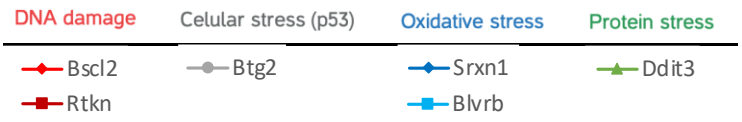


Figure 5: ToxTracker results for 1,2-dibromoethane and 8-hydroxyquinoline. **(A)** Activation of the six different ToxTracker reporters following exposure to increasing concentration of the test compound. The Bscl2 and Rtkn reporters indicate genotoxicity, Srxn1 and Blvrb are induced by oxidative stress, Btg2 is associated with the p53 tumor suppressor response and Ddit3 is induced by protein misfolding. **(B)** Cytotoxicity of the compounds is determined by relative cell count in cultures exposed to the compound and their related vehicle control cultures.

39. Overall, all tested non-genotoxic compounds in the validation trial were correctly predicted as non-genotoxic in ToxTracker. In a weighted approach, none of these compounds induced the Bscl2 and Rtkn genotoxicity reporters (Table 12). However, approximately 50% of the tested non-genotoxic compounds induced oxidative stress or protein unfolding which have been associated with carcinogenicity (15,47). All compounds that were classified as non-genotoxic in ToxTracker were negative in the Ames bacterial mutation assay. However, a number of compounds that were predicted non-genotoxic in ToxTracker (no activation of Bscl/Rtkn reporters) have been reported to induce positive results in the *in vitro* MN or CA assay (16,18). Occasionally, positive results from these *in vitro* clastogenicity assays do not correctly predict *in vivo* genotoxicity. Various reasons for this discrepancy have been proposed, including misleading *in vitro* positive responses caused by high levels of cytotoxicity (48). Also, indirect genotoxicity caused by high levels of oxidative stress can cause positive results in the *in vitro* MN assay but are often not

observed *in vivo* due to lower *in vivo* exposure levels and more efficient anti-oxidant systems (48). For example, lead acetate was shown to induce DNA strand breaks *in vitro* due to oxidative stress and also tert-butyl hydroquinone gave positive results in the *in vitro* CA assay (43,49). In this ToxTracker validation trial, the different laboratories reported high levels of oxidative stress for these compounds. Based on the dose response curves for the genotoxicity reporters (Bscl2/Rtkn) and oxidative stress (Srxn1/Blvrb), oxidative stress induction appeared to be the primary mechanism of toxicity for these compounds (Figure 5). In the validation trial, lead acetate and tert-butyl hydroquinone were classified as non-genotoxic although 1 lab reported a positive result for the Rtkn reporter for DNA strand breaks after exposure to lead acetate and tert-butyl hydroquinone. Also, induction of protein damage has been associated with induction of genotoxic effects, primarily *in vitro* (48). In the ToxTracker trial, tunicamycin and p-nitrophenol were classified as non-genotoxic but both compounds activated the Ddit3 reporter for protein unfolding. Tunicamycin is non-genotoxic *in vivo*, but induced MN *in vitro*, p-nitrophenol in positive in the *in vitro* CA and MN assays. Together these examples indicate that information about the genotoxic and non-genotoxic effects of compounds can be valuable to more accurately predict the *in vivo* genotoxic effects of compounds. The genotoxic MoA information can also be used to explain discrepancies between various *in vitro* and *in vivo* genotoxicity assays in a WoE approach.



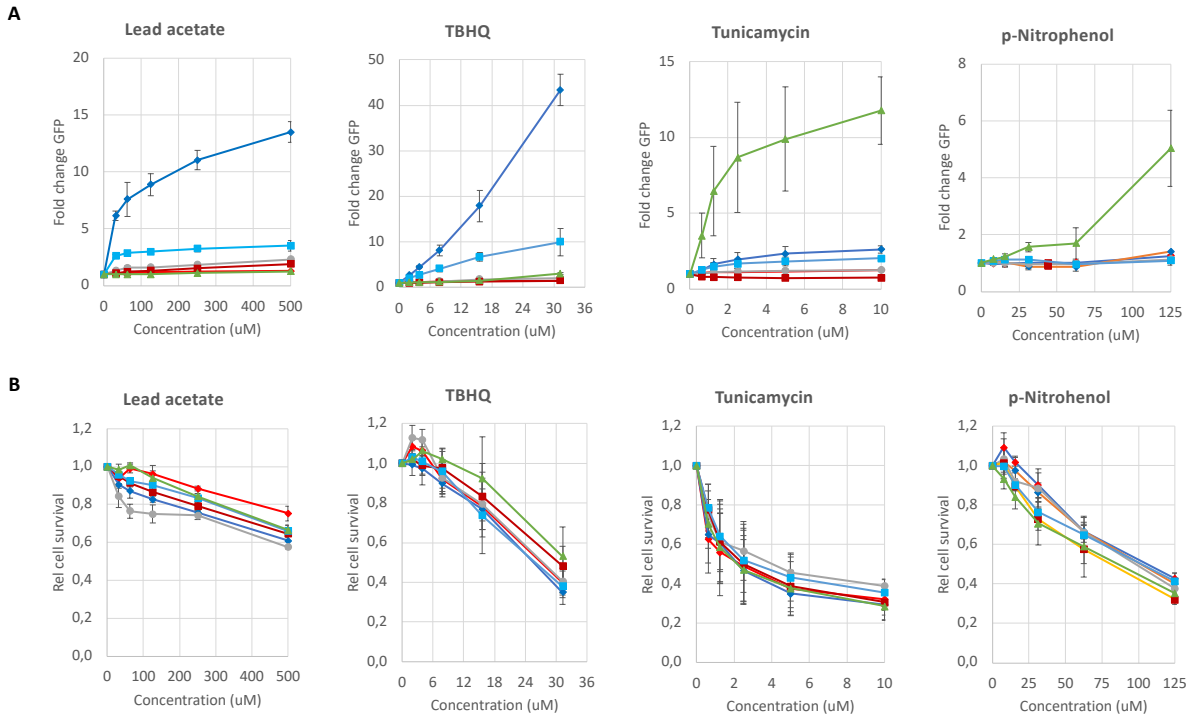


Figure 5: ToxTracker results for the non-genotoxic compounds lead acetate, tert-butyl hydroquinone, tunicamycin and p-nitrophenol. **(A)** Activation of the six different ToxTracker reporters following exposure to increasing concentration of the test compound. The Bscl2 and Rtkn reporters indicate genotoxicity, Srxn1 and Blvrb are induced by oxidative stress, Btg2 is associated with the p53 tumor suppressor response and Ddit3 is induced by protein misfolding. **(B)** Cytotoxicity of the compounds is determined by relative cell count in cultures exposed to the compound and their related vehicle control cultures.

40. To further explore the contribution of the different reporter genes in the genotoxicity prediction, the ToxTracker results were compared to outcomes of the standard *in vitro* and *in vivo* genotoxicity assays (Table 13). For this comparison, a number of compounds were selected for which data are available in the public domain that allow a WoE-based classification as compounds with MoAs involving either oxidative stress (blue) or unfolded protein response (green). For all ten compounds that were expected to induce oxidative stress, activation of the oxidative stress reporters (Srxn1/Blvrb) was observed by the laboratories. Four compounds that have been shown to induce the unfolded protein response, activated the Ddit3 reporter in ToxTracker. Importantly, nearly all of the selected compounds were predicted to be non-genotoxic in ToxTracker and were also negative in the standard *in vivo* genotoxicity assays. In contrast, many of these compounds were classified as genotoxic in at least one of the standard *in vitro* genotoxicity assays (Ames, MN, CA). The MoA information that is obtained from the ToxTracker assay could help to gain mechanistic insight into the hazardous properties of compounds and to improve the *in vivo* genotoxicity prediction.

Table 13: Comparison between ToxTracker and the standard in vitro and in vivo genotoxicity assays for compounds with an oxidative stress or protein reactive MoA.

	Compound	CAS number	In vitro				In vivo			ToxTracker	
			Ames	MLA	MN	CA	MN	CA	TgR	Genotoxic	MoA
Group I: Genotoxic carcinogens											
18	Cadmium Chloride	10108-64-2	E		P	P	P	P		N	Oxidative stress
22	4-nitroquinoline-1-oxide	56-57-5	P	P	P	P	P	P	P	P	DNA reactive, oxidative stress
Group II: Genotoxic non-carcinogens											
25	p-Phenylenediamine 2HCl	624-18-0	P	P	P	P	N			P	DNA reactive, oxidative stress
26	8-Hydroxyquinoline	148-24-3	P			P	N			P	Indirect genotoxin, oxidative stress, protein reactive
32	Phenol	108-95-2	N		P	P	P	N		P	Indirect genotoxin, oxidative stress
Group III: Non-genotoxic carcinogens											
34	Lead (ii) acetate trihydrage	6080-56-4	N		P	E	E	P		E	Oxidative stress
35	2-Phenylphenol sodium salt	6152-33-6	P			E	N	N		N	Oxidative stress, protein reactive
38	Cyclosporin A (CsA)	59865-13-3	N				N			N	Protein reactive
Group IV: Non-genotoxic non-carcinogens											
43	Tunicamycin	11089-65-9	N		P		N			N	Protein reactive
44	p-Nitrophenol (4-nitrophenol)	100-02-7	N		E	P	N			N	Protein reactive
45	Phenanthrene	85-01-8	P		E	E				N	Oxidative stress, protein reactive
46	Tertiarybutylhydroquinone	1948-33-0	N		P	P	N	N		N	Oxidative stress, protein reactive
54	Chlorpheniramine maleate	113-92-8	N	P		P	N			N	Oxidative stress
58	Allyl alcohol	107-18-6	P	P		P	N			N	Oxidative stress

12. Within-lab and between-lab reproducibility for genotoxicity predictions

41. One of the primary objectives of the ToxTracker ring trial was to establish the transferability and reproducibility of the assay. We first focused on the reproducibility of the genotoxicity prediction in ToxTracker. For each of the participating laboratories, the WLR was determined. Every compound was tested in three independent repeat experiments for activation of the Bscl2-GFP and Rtkn-GFP genotoxicity reporters. For each of these biomarkers, the results from the repeat experiments were analyzed for their acceptability according to the criteria set in the ToxTracker protocol, with expert judgement where appropriate. Next, from every acceptable experiment, the positive or negative classifications for the different reporters were compared (Table 14 provides an example for one of the laboratories). The experiments were considered reproducible if the laboratory came to the same conclusion in the three independent repeat tests. For the example shown in Table 14, the reproducibility was 96.7% for the genotoxicity classification. For some compounds, e.g. phenol, lead acetate and tert-butyl hydroquinone, the three repeat experiments gave slightly different results but the classification of the compounds was identical between the experiments.

Table 14: Example of the WLR of the two genotoxicity reporters in ToxTracker within one of the validation laboratories

			Genotoxicity			
			Bscl2 Result -S9	Bscl2 Result +S9	Rtkn Result -S9	Rtkn Result +S9
Code	Compound	Cas#				
1	Etoposide	33419-42-0	+++	+++	+++	+++
2	Mitomycin C	50-07-7	+++	+++	+++	+++
3	Cisplatin	15663-27-1	+++	+++	+++	+++
4	1,2- Dimethylhydrazine	306-37-6	+++	+++	+++	+++
6	Cyclophosphamide	6055-19-2	---	+++	---	+++
13	Busulfan	55-98-1	+++	+++	+++	+++
14	Ethyl methanesulfonate	62-50-0	+++	+++	+++	+++
19	Dimethylnitrosamine	62-75-9	---	---	---	---
21	o-Anisidine	90-04-0	---	+++	+++	+++
23	6-Mercaptopurine	50-44-2	---	(+)-(+)	+++	+-+
25	p-Phenylenediamine 2HCl	624-18-0	+++	+++	+++	+++
27	9-Aminoacridine	90-45-9	---	---	+++	+++
29	3-Nitropropionic acid	504-88-1	+++	+++	+++	+++
31	5-fluorouracil	51-21-8	-++	-++	-++	+++
32	Phenol	108-95-2	---	-(+)-	-++	+++
34	Lead (ii) acetate	6080-56-4	-(+)(+)	---	(+)++	---
38	Cyclosporin A	59865-13-3	---	---	---	---
40	Diethanolamine	111-42-2	---	---	---	---
42	Melamine	108-78-1	+++	+++	+++	+++
43	Tunicamycin	11089-65-9	---	---	---	---
45	Phenanthrene	85-01-8	---	---	---	---
46	Tertiarybutylhydroquinone	1948-33-0	(+)-(+)	(+)(+)-	(+)+-	+++
48	Vanilin	121-33-5	---	---	---	+++
49	Erythromycin stearate	114-07-8	---	---	---	---
51	o-anthranilic acid	118-92-3	---	---	---	---
52	Tolbutamide	64-77-7	---	---	---	---
53	2-ethyl-1,3-hexanediol	94-96-2	---	---	---	---
54	Chlorpheniramine maleate	113-92-8	---	---	---	---
57	D-mannitol	69-65-8	---	---	---	---
62	Cyclohexanone	108-94-1	---	---	---	---

42. For every laboratory, the WLR was calculated. We determined the WLR for the genotoxicity classification of the compounds, meaning the activation of the Bscl2 and Rtkn genotoxicity reporters was assessed in the absence and presence of S9-mix to classify the compounds as genotoxic. The reproducibility of genotoxicity classification for every compound was determined between repeat experiments (Table 14). The number of compounds that were tested in the different laboratories varied between 24 and 30. Results were considered reproducible if the laboratory gave the compound the same classification in the three repeat experiments. Non-reproducible (in Table 15) means that at least in one experiment, the laboratory came to a different classification of the compounds. Overall, the WLR in 6 of the 7 participating laboratories varied between 80% and 96.7%. However, Laboratory 4 had some challenges with accurate measurements of cell numbers which resulted in an overestimation of the cytotoxicity of compounds. Following the data acceptance criteria for

ToxTracker, data obtained at concentrations that induce >75% cytotoxicity should be discarded from analysis. The cytotoxicity assessment issues in lab 4 therefore resulted in a relatively high number of inconclusive tests which led to lack of reproducibility and negatively impacted their WLR calculations.

Table 15: Within-laboratory reproducibility for the Bscl2 and Rtkn genotoxicity reporters in the seven ToxTracker validation laboratories.

Lab	Tested compounds	Reproducible	Non-reproducible	WLR
1	30	29	1	96,7%
2	24	22	2	91,7%
3	25	23	2	92,0%
4	26	19	7	73,1%
5	24	20	4	83,3%
6	30	24	6	80,0%
7	27	22	5	81,5%

43. Finally, also the BLR in the ToxTracker ring trial for the prediction of genotoxicity was determined. For this, the classifications of the tested chemicals for their induction of the Bscl2 and Rtkn genotoxicity reporters were compared between the participating laboratories. For each lab, an overall classification was made from the three repeat experiments. These overall classifications were compared between laboratories to establish the BLR. As an example, the ToxTracker results for etoposide from the three laboratories are summarized in Table 16. In this example, all three labs classified etoposide as genotoxic compound in all three biological repeat experiments. Results were reproducible with each lab as well as between labs.

Table 16: Example of the between-laboratory reproducibility for the activation of the different genotoxicity reporters in ToxTracker following exposure to etoposide (compound 1).

	Bscl2							
	-S9	+S9	Final call -S9	Final call +S9	Overall Final call	No. Valid runs	WLR	BLR
LAB 1	PPP	PPP	P	P	P	3	Yes	
LAB 2	PPP	NNN	P	N	P	3	Yes	Yes
LAB 3	PPP	P(P)(P)	P	P	P	3	Yes	

	Rtkn							
	-S9	+S9	Final call -S9	Final call +S9	Overall Final call	No. Valid runs	WLR	BLR
LAB 1	PPP	PPP	P	P	P	3	Yes	
LAB 2	PPP	PPN	P	P	P	3	Yes	Yes
LAB 3	PPP	PPP	P	P	P	3	Yes	

44. For the genotoxicity BLR calculation, the results for the Bscl2 and Rtkn reporters were combined as a positive call for each of these biomarkers would lead to a positive genotoxicity classification for a compound. The BLR was determined for 59 compounds in the ring trial for which acceptable data from at least two laboratories were available. Compounds for which only acceptable data was obtained from one laboratory were excluded from the BLR calculations. The BLR for the genotoxicity predictions in the ToxTracker ring trial between the seven validation laboratories was 83.1%.

13. WLR and BLR for MoA assessment from all ToxTracker reporters

45. There are currently no consolidated databases available with compounds that specifically induce oxidative stress or the unfolded protein response. It is therefore not possible to calculate the sensitivity and specificity of the ToxTracker reporters for prediction of oxidative stress (Srxn1/Blvrb) or protein damage (Ddit3). We therefore focused on the transferability and reproducibility of all six ToxTracker reporters by the different laboratories. All compounds were tested for activation of the biomarkers that indicate induction of DNA damage, oxidative stress, protein damage and p53-associated cellular stress. For each of these biomarkers, the results from the repeat experiments were analyzed and the positive or negative classifications for each of the reporters were compared (Table 17 provides an example for one of the laboratories). The experiments were considered reproducible if the laboratory came to the same conclusion in the different repeats. For the example shown in Table 17, the reproducibility varied between 96.7% for induction of DNA damage (activation of Bscl2 and/or Rtkn), protein damage (Ddit3) and p53-associated cellular stress (Btg2), and 100% for oxidative stress (Srxn1 and/or Blvrb). For some compounds, the three repeat experiments gave slightly different results, but the classification of the compounds was identical between the experiments. For example, cyclophosphamide was negative in two repeats for induction of oxidative stress in the absence of S9-mix, but in the third repeat a weak positive (+) was recorded. Nevertheless, the overall call for oxidative stress was negative according to the ToxTracker prediction model (Table 2 and 3). In case that induction of a reporter was different between repeats, but the results -S9

and +S9 together resulted in similar calls in the three repeats, the results were indicated as reproducible.

Table 17: Example of the intra-lab reproducibility of the six different ToxTracker reporters within one of the validation laboratories

			Genotoxicity				Oxidative stress				Protein damage		Cellular stress	
			Bscl2	Bscl2	Rtkn	Rtkn	Srxn1	Srxn1	Blvrb	Blvrb	Ddit	Ddit	Btg2	Btg2
Code	Compound	Cas#	Result -S9	Result +S9	Result -S9	Result +S9	Result -S9	Result +S9	Result -S9	Result +S9	Result -S9	Result +S9	Result -S9	Result +S9
1	Etoposide	33419-42-0	+++	+++	+++	+++	+++	+++	(+)(+)(+)	--(+)	---	---	+++	+++
2	Mitomycin C	50-07-7	+++	+++	+++	+++	+++	+++	+++	+++	---	---	+++	+++
3	Cisplatin	15663-27-1	+++	+++	+++	+++	+++	+++	+++	+++	---	---	+++	+++
4	1,2- Dimethylhydrazine	306-37-6	+++	+++	+++	+++	+++	+++	+++	+++	---	---	+++	+++
6	Cyclophosphamide	6055-19-2	---	+++	---	+++	--(+)	+++	---	+++	---	---	---	+++
13	Busulfan (Myleran)	55-98-1	+++	+++	+++	+++	+++	+++	+++	+++	---	---	+++	(+)++
14	Ethyl methanesulfonate (EMS)	62-50-0	+++	+++	+++	+++	+++	+++	+++	+++	---	---	+++	+++
19	Dimethylnitrosamine (N-nitrosodimethy	62-75-9	---	---	---	---	---	---	---	---	---	---	---	---
21	o-Anisidine	90-04-0	---	+++	+++	+++	+++	+++	+++	+++	---	---	+++	+++
23	6-Mercaptopurine	50-44-2	---	(+)-(+)	+++	+-+	+++	+(+)+	-(+)(+)	---	---	---	+++	+++
25	p-Phenylenediamine 2HCl	624-18-0	+++	+++	+++	+++	+++	+++	+++	+++	---	---	+++	+++
27	9-Aminoacridine	90-45-9	---	---	+++	+++	+ + +	+ + +	- - -	- - -	---	---	+++	+++
29	3-Nitropropionic acid	504-88-1	+++	+++	+++	+++	+++	+++	+++	+++	---	---	+++	+++
31	5-fluorouracil	51-21-8	-++	-++	-++	+++	-++	+++	-+-	-+(+)	---	---	-++	+++
32	Phenol	108-95-2	---	-(+)-	-++	+++	+++	+++	---	+++	---	---	+-+	+++
34	Lead (ii) acetate trihydrage available	6080-56-4	-(+)(+)	---	(+)++	---	+++	+++	+++	+--	---	---	+++	---
38	Cyclosporin A (CsA)	59865-13-3	---	---	---	---	+++	+++	(+)++	+++	+++	+++	---	---
40	Diethanolamine (DEA or DEOA)	111-42-2	---	---	---	---	---	---	---	---	---	---	---	---
42	Melamine	108-78-1	+++	+++	+++	+++	---	---	---	---	---	---	---	---
43	Tunicamycin	11089-65-9	---	---	---	---	+++	+-+	+(+)+	+(+)-	+++	+++	---	---
45	Phenanthrene	85-01-8	---	---	---	---	+-+	+++	---	(+)-+	--+	+++	---	---
46	Tertiarybutylhydroquinone	1948-33-0	(+)-(+)	(+)(+)-	(+)+-	+++	+++	+++	+++	+++	+++	+- +	+++	+++
48	Vanilin	121-33-5	---	---	---	+++	+-+	--(+)	---	+++	---	---	+--	---
49	Erythromycin (Erythromycin stearate 6-	114-07-8	---	---	---	---	++-	+++	-(+)-	(+)--	+++	---	---	---
51	o-anthranilic acid	118-92-3	---	---	---	---	---	---	---	---	---	---	---	---
52	Tolbutamide	64-77-7	---	---	---	---	+++	+++	---	---	+++	++-	---	---
53	2-ethyl-1,3-hexanediol	94-96-2	---	---	---	---	+++	+++	---	---	---	+--	---	---
54	Chlorpheniramine maleate	113-92-8	---	---	---	---	+++	+++	+++	-++	---	---	---	---
57	D-mannitol	69-65-8	---	---	---	---	---	---	---	---	---	---	---	---
62	Cyclohexanone	108-94-1	---	---	---	---	---	---	---	---	---	---	---	---

46. In addition to reproducibility of the genotoxicity prediction (discussed above), WLR was also determined for the 4 other reporter genes following the same criteria (Table 18). The WLR was comparable to that seen for Bscl2 and Rtkn for 6 of the 7 labs, with Laboratory 4 again showing low WLR values due to the cytotoxicity issues discussed above. The overall WLR (average of all reporters) varies from 97.5% for the lab with the best overall performance and 71.1% for the lab with the lowest reproducibility. Together, these WLR calculations, together with the successful proficiency tests during the second phase of the validation trial confirm the excellent transferability of the ToxTracker assay.

Table 18: Within-laboratory reproducibility for the various ToxTracker reporters in the different validation laboratories.

	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7
DNA damage	96,7	91,7	92,0	73,1	83,3	80,0	81,5
Oxidative stress	100,0	75,0	73,3	77,8	91,7	70,0	81,5
Protein damage	96,7	91,7	87,5	74,1	100,0	86,7	75,0
p53 activation	96,7	70,8	82,6	59,3	87,5	83,3	81,5

47. Finally, also the BLR in the ToxTracker validation trial for all reporters was determined. For this, the classifications of the tested chemicals for their induction of DNA damage, oxidative stress, protein damage and p53-associated cellular stress were compared between the participating laboratories. For each lab, an overall classification was made for every endpoint in ToxTracker from the three repeat experiments. These overall classifications were compared between laboratories to establish the BLR. As an example, the ToxTracker results for etoposide from the three laboratories are summarized in Table 19. Five out of six ToxTracker reporters gave a similar result in the three laboratories. Only the result for the Blvrb reporter was not reproducible in this example.

Table 19: Example of the between-laboratory reproducibility for the activation of the different ToxTracker reporters following exposure to etoposide.

	Bscl2							
	-S9	+S9	Final call -S9	Final call +S9	Overall Final call	No. Valid runs	WLR	BLR
LAB 1	PPP	PPP	P	P	P	3	Yes	
LAB 2	PPP	NNN	P	N	P	3	Yes	Yes
LAB 3	PPP	P(P)(P)	P	P	P	3	Yes	

	Rtkn							
	-S9	+S9	Final call -S9	Final call +S9	Overall Final call	No. Valid runs	WLR	BLR
LAB 1	PPP	PPP	P	P	P	3	Yes	
LAB 2	PPP	PPN	P	P	P	3	Yes	Yes
LAB 3	PPP	PPP	P	P	P	3	Yes	

	Srxn1							
	-S9	+S9	Final call -S9	Final call +S9	Overall Final call	No. Valid runs	WLR	BLR
LAB 1	PPP	PPP	P	P	P	3	Yes	
LAB 2	PPP	N(P)N	P	N	P	3	Yes	Yes
LAB 3	PPP	NNP	P	N	P	3	Yes	

	Blvrb							
	-S9	+S9	Final call -S9	Final call +S9	Overall Final call	No. Valid runs	WLR	BLR
LAB 1	(P)(P)(P)	NN(P)	E	N	E	3	Yes	
LAB 2	PNN	IPN	N	E	E	3	No	No
LAB 3	NNN	NNN	N	N	N	3	Yes	

	Ddit							
	-S9	+S9	Final call -S9	Final call +S9	Overall Final call	No. Valid runs	WLR	BLR
LAB 1	NNN	NNN	N	N	3	N	Yes	
LAB 2	NNN	NNN	N	N	3	N	Yes	Yes
LAB 3	NNN	NNN	N	N	3	N	Yes	

	Btg2							
	-S9	+S9	Final call -S9	Final call +S9	Overall Final call	No. Valid runs	WLR	BLR
LAB 1	PPP	PPP	P	P	P	3	Yes	
LAB 2	PPP	NPP	P	P	P	3	Yes	Yes
LAB 3	PPP	PPP	P	P	P	3	Yes	

48. For the BLR calculation, the results for the Bscl2 and Rtkn reporters were combined since a positive call for either of these biomarkers would lead to a positive genotoxicity classification for a compound. The same approach was used for the Srxn1and Blvrb oxidative stress reporters. The BLR was determined for 59 compounds in the ring trial for which acceptable data from at least two laboratories were available. The BLR was calculated for the different toxicological endpoints in ToxTracker. The BLR in the ToxTracker validation trial for the seven validation laboratories varied between 83% for the genotoxicity predictions and 71% for oxidative stress (Table 20). The overall reproducibility of the classification of genotoxic compounds was 91%. The overall reproducibility of predicting protein damage and p53-associated cellular stress was comparable for the genotoxic and non-genotoxic compounds.

Table 19: Between-lab reproducibility for the different toxicological endpoints that are assessed in ToxTracker.

	DNA damage	Oxidative stress	Protein damage	Cell stress
	Bscl2 / Rtkn	Srxn1 / Blvrb	Ddit3	Btg2
BLR	83,1	71,0	82,5	78,3

14. Availability of data for expert review

49. All results from the ToxTracker ring trial are available for review. The test results are collected in a large database, but this is also accessible through an excel spreadsheet. Full statistical analysis has been performed on the primary data sets. Publications about the development and technical validation of the ToxTracker assay have been published by Toxys and may be made available for expert review upon request. Various case studies using ToxTracker for genotoxicity testing and MoA assessment of pharmaceutical, (agro)chemical, cosmetic and flavor/fragrance compounds have been published in peer-reviewed journals.

15. Discussion and learnings

50. The interlaboratory validation of the ToxTracker assay was performed according to OECD guidance document 34 wherever reasonably possible. The project was coordinated by the experts in the VMT. Toxys, the developer of ToxTracker, provided technical support to the project, but was not involved in selection of the compounds for the validation trial or in data analysis. Also coding and distribution of the compounds to the participating labs was done by the VMT, excluding Toxys. The participating laboratories received the blinded compounds and instructions how to perform the tests. Data analysis was started when all the laboratories provided their results. Data analysis was performed by the VMT. First the results were analyzed for their acceptability, based on the data acceptance criteria that were defined by the VMT and described in the validation protocol. Next the acceptable results for the tested compounds were analyzed for the induction of genotoxicity. This was an important first step since information on MoA would only be valuable if it was shown that ToxTracker could reliably distinguish between genotoxic and non-genotoxic substances. The WLR and BLR was determined, as well as the overall sensitivity and specificity for identification of genotoxic compounds. Finally, also the additional reporters that provide further insight into the MoA of

genotoxic compounds were analyzed. Throughout the data analysis, compounds remained blinded to prevent any bias in the analysis. After sharing and discussing the results from the validation trial with the full validation consortium and approval of the data, the compounds were decoded.

51. During the training phases of the validation trial, a number of changes and clarifications were made to the ToxTracker protocol. The most important modification was a change in the S9 metabolization protocol. ToxTracker relies on S9 rat liver extract for metabolization of compounds. Originally, the standard S9 protocol that is also used in the Ames and in vitro MN assay was included in ToxTracker (7,8). In this protocol, cells are exposed to the compounds in presence of 1% S9-mix for 3 hours, followed by a 21 hour culture period without S9-mix before analysis of the fluorescent reporters. Exposure times are limited to 3 hours because of the potential toxicity of S9-mix. Although when using this S9 protocol, genotoxic compounds are effectively metabolized and correctly identified as genotoxic by ToxTracker, the recovery time after exposure resulted in a strong reduction in signals for oxidative stress and protein damage induction. To improve the sensitivity for detection of all cellular responses in ToxTracker, the S9 protocol for ToxTracker was optimized by Toxys. In this improved protocol, cells are exposed to 0.25% S9-mix for 24 hours continuously and ToxTracker reporter activation is analyzed immediately after exposure without a recovery period. This improved protocol was also implemented in the ToxTracker validation protocol during the validation trial. The laboratories were requested to first test the genotoxic compound benzo[a]pyrene during the second phase of the validation (proficiency testing) using the original S9 protocol (Figure 6A). Benzo[a]pyrene was correctly classified as genotoxic by the laboratories. After adoption of the updated S9 protocol by the VMT, all labs were required to retest benzo[a]pyrene. With the new S9 protocol, the genotoxicity of benzo[a]pyrene was confirmed, and also oxidative stress induction was readily detected (figure 6B). The updated S9 protocol was therefore applied throughout the third phase of the validation trial.

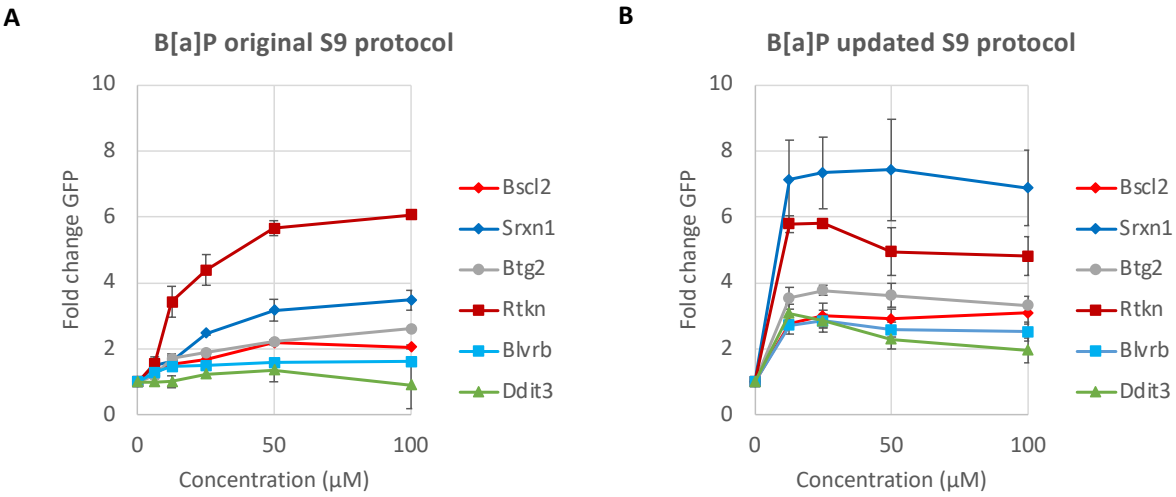


Figure 6: Genotoxicity of benzo[a]pyrene in ToxTracker in the original and updated S9 metabolization protocol **(A)** Activation of the six different ToxTracker reporters following exposure to increasing concentration of the test compound. The Bscl2 and Rtkn reporters indicate genotoxicity, Srxn1 and Blvrb are induced by oxidative stress, Btg2 is associated with the p53 tumor suppressor response and Ddit3 is induced by protein misfolding. **(B)** Cytotoxicity of the compounds is determined by relative cell count in cultures exposed to the compound and their related vehicle control cultures.

52. During the second phase of the validation project, a few aspects in the ToxTracker protocol needed clarification, mostly related to compound handling and stock solution preparation. Also, there were a number of questions about quality control and data acceptance. Therefore, after the second phase (proficiency testing), a number of instructions on preparing compound solution, establishing precipitation and quality controls for the assay were included in the ToxTracker protocol by the validation team. These instructions helped the laboratories to increase the reproducibility of their repeat experiments. The most important reason for variation between repeats in the ToxTracker validation was the accurate measurement of cytotoxicity which could unnecessarily invalidate experiments. In addition, clear instructions were provided how to make the compound stock solutions and how to handle compounds that did not dissolve. During the proficiency testing, it became apparent that laboratories had different approaches to establish precipitation of compounds in the cell culture plates. Clear instructions how to determine the maximum soluble concentrations were added to the protocol by the VMT.

53. After completion of the ToxTracker validation trial and analysis of the results, the VMT came to a number of conclusions and learnings for future improvements of ToxTracker. A recommendation for future applications of ToxTracker would be to improve the instructions and quality controls for relative cell counting using the flow cytometer. Another learning from the ring trial was that the positive control compound Aflatoxin B1, included to ensure proper activity of S9-mix, was not very stable and was a source for variation. A

modification to the ToxTracker protocol to use cyclophosphamide as an alternative positive control compound requiring metabolic activation has already been made.

16. Summary and conclusions

54. ToxTracker is a high content *in vitro* reporter assay which has been shown to be very useful for the accurate prediction of *in vivo* genotoxicity. By combining various reporters that indicate different toxicological effects relevant for genetic toxicology, ToxTracker has the advantage that it can provide insight into the mode of action of genotoxic and non-genotoxic chemicals. The regulatory need and applications for ToxTracker are outlined in this document. In order to investigate how ToxTracker may complement the standard battery of *in vitro* genotoxicity assays, a comprehensive interlaboratory validation trial of the ToxTracker assay was organized. The validation was conducted using the principles outlined in OECD GD 34. The primary objectives of the validation were to establish the transferability and reproducibility of the assay, and to confirm the ability of ToxTracker to correctly classify compounds as genotoxic. In addition, the reproducibility to predict the genotoxic MoA was investigated and how this information can be applied to improve in vitro prediction of in vivo genotoxicity. During the validation trial, ToxTracker was successfully installed in the laboratories of the seven partner laboratories. A limited proficiency test confirmed the ability of the laboratories to perform the assay. Although no major problems occurred, a number of improvements was made to the ToxTracker protocol. During the validation trial, the seven laboratories tested 64 chemicals (32 expected to be positive and 32 expected to be negative) that together cover a broad spectrum of chemical spaces. Also metabolic activation of chemicals by liver enzymes was included in the study. Each compound was tested in three laboratories. During the validation trial, we determined the within and between-lab reproducibility of ToxTracker. The WLR varied between 80% and 96.7% for 6/7 of the different laboratories confirming the good transferability of the assay when cytotoxicity is accurately assessed. One laboratory suffered from some technical issues, resulting in a lower WLR of 73.1%. The BLR for genotoxicity classification of chemicals was in the region of 83%. The interlaboratory validation confirmed the accuracy of ToxTracker to correctly identify genotoxic compounds with a sensitivity of 87% and a specificity of 90%. Together, from this validation trial we concluded that ToxTracker is a robust *in vitro* assay for the accurate prediction of *in vivo* genotoxicity. With information on the MoA of chemicals that is provided by the assay, ToxTracker would be a valuable addition to the battery of genotoxicity assays that is applied for regulatory applications.

17. References

1. ICH. International conference on harmonisation of technical requirements for registration of pharmaceuticals for human use ICH harmonised tripartite guideline guidance on genotoxicity testing and data interpretation for pharmaceuticals intended for human use S2(R1). 2011.
2. https://echa.europa.eu/guidance-documents/guidance-on-information-requirements-and-chemical-safety-assessment. ECHA GD for chemical safety assessment.
3. Hardy A, Benford D, Halldorsson T, Jeger M, Knutsen HK, More S, et al. Clarification of some aspects related to genotoxicity assessment. EFSA Journal. 2017 Dec;15(12).
4. SCCS (Scientific Committee on Consumer Safety), SCCS Notes of Guidance for the Testing of Cosmetic Ingredients and their Safety Evaluation 11th revision, 30-31 March 2021, SCCS/1628/21. 2021.
5. Thomas R. The US Federal Tox21 Program: A strategic and operational plan for continued leadership. ALTEX. 2018;163–8.
6. How to use alternatives to animal testing to fulfil your information requirements for REACH registration [Internet]. 2016 [cited 2022 Sep 28]. Available from: https://echa.europa.eu/documents/10162/17250/practical_guide_how_to_use_alternatives_en.pdf/148b30c7-c186-463c-a898-522a888a4404
7. Hendriks G, Atallah M, Morolli B, Calléja F, Ras-Verloop N, Huijskens I, et al. The ToxTracker assay: Novel GFP reporter systems that provide mechanistic insight into the genotoxic properties of chemicals. Toxicological Sciences. 2012 Jan 1;125(1):285–98.
8. Hendriks G, Derr RS, Misovic B, Morolli B, Calléja FMGR, Vrieling H. The Extended ToxTracker Assay Discriminates Between Induction of DNA Damage, Oxidative Stress, and Protein Misfolding. Toxicological Sciences. 2016 Mar 1;150(1):190–203.
9. Hendriks G, Atallah M, Raamsman M, Morolli B, van der Putten H, Jaadar H, et al. Sensitive DsRed fluorescence-based reporter cell systems for genotoxicity and oxidative stress assessment. Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis. 2011 May 10;709–710:49–59.
10. Hendriks G, Derr RS, Misovic B, Morolli B, Calléja FMGR, Vrieling H. The Extended ToxTracker Assay Discriminates

Between Induction of DNA Damage, Oxidative Stress, and Protein Misfolding. Toxicological Sciences. 2016;150(1):190–203.

11. Kirsch-Volders M, Vanhauwaert A, Eichenlaub-Ritter U, Decordier I. Indirect mechanisms of genotoxicity. Toxicol Lett. 2003 Apr;140–141:63–74.

12. Lynch AM, Eastmond D, Elhajouji A, Froetschl R, Kirsch-Volders M, Marchetti F, et al. Targets and mechanisms of chemically induced aneuploidy. Part 1 of the report of the 2017 IWGT workgroup on assessing the risk of aneugens for carcinogenesis and hereditary diseases. Mutat Res Genet Toxicol Environ Mutagen [Internet]. 2019 Nov 1 [cited 2022 Jul 13];847. Available from: https://pubmed.ncbi.nlm.nih.gov/31699346/

13. Brandsma I, Moelijker N, Derr R, Hendriks G. Aneugen Versus Clastogen Evaluation and Oxidative Stress-Related Mode-of-Action Assessment of Genotoxic Compounds Using the ToxTracker Reporter Assay. Toxicological Sciences. 2020 Sep 1;177(1):202–13.

14. Nohmi T. Thresholds of Genotoxic and Non-Genotoxic Carcinogens. Toxicol Res. 2018 Oct 15;34(4):281–90.

15. Hendriks G, van de Water B, Schoonen W, Vrieling H. Cellular-signaling pathways unveil the carcinogenic potential of chemicals. Journal of Applied Toxicology. 2013;33(6):399–409.

16. Kirkland D, Kasper P, Martus HJ, Müller L, van Benthem J, Madia F, et al. Updated recommended lists of genotoxic and non-genotoxic chemicals for assessment of the performance of new or improved genotoxicity tests. Mutat Res Genet Toxicol Environ Mutagen [Internet]. 2016 Jan 1 [cited 2022 Jul 14];795:7–30. Available from: https://pubmed.ncbi.nlm.nih.gov/26774663/

17. Wills JW, Halkes-Wellstead E, Summers HD, Rees P, Johnson GE. Empirical comparison of genotoxic potency estimations: The in vitro DNA-damage ToxTracker endpoints versus the in vivo micronucleus assay. Mutagenesis. 2021 Jul 1;36(4):311–20.

18. Fowler P, Smith K, Young J, Jeffrey L, Kirkland D, Pfuhler S, et al. Reduction of misleading (“false”) positive results in mammalian cell genotoxicity assays. I. Choice of cell type. Mutat Res [Internet]. 2012 Feb 18 [cited 2022 Jul 22];742(1–2):11–25. Available from: https://pubmed.ncbi.nlm.nih.gov/22138618/

19. Kakehashi A, Wei M, Fukushima S, Wanibuchi H. Oxidative Stress in the Carcinogenicity of Chemical Carcinogens. Cancers (Basel) [Internet].

2013 Dec 1 [cited 2023 Mar 27];5(4):1332. Available from: /pmc/articles/PMC3875942/

20. Madden E, Logue SE, Healy SJ, Manie S, Samali A. The role of the unfolded protein response in cancer progression: From oncogenesis to chemoresistance. Biol Cell [Internet]. 2019 Jan 1 [cited 2023 Mar 27];111(1):1–17. Available from: https://onlinelibrary.wiley.com/doi/full/10.1111/boc.201800050

21. Allemang A, Lester C, Roth T, Pfuhler S, Peuschel H, Kosemund K, et al. Assessing the genotoxicity and carcinogenicity of 2-chloroethanol through structure activity relationships and in vitro testing approaches. Food and Chemical Toxicology. 2022 Oct;168:113290.

22. Smart DE, Bozhilova S, Miazzi F, Haswell LE, Gaca MD, Thorne D, et al. Application of ToxTracker for the toxicological assessment of tobacco and nicotine delivery products. Toxicol Lett [Internet]. 2022 Jan 19 [cited 2022 Jan 26]; Available from: http://www.ncbi.nlm.nih.gov/pubmed/35065211

23. Mesnage R, Ibragim M, Mandrioli D, Falcioni L, Tibaldi E, Belpoggi F, et al. Comparative toxicogenomics of glyphosate and Roundup herbicides by mammalian stem cell-based genotoxicity assays and molecular profiling in Sprague-Dawley rats. Toxicol Sci [Internet]. 2021 Nov 29 [cited 2022 Jan 26]; Available from: http://www.ncbi.nlm.nih.gov/pubmed/34850229

24. White PA, Long AS, Johnson GE. Quantitative Interpretation of Genetic Toxicity Dose-Response Data for Risk Assessment and Regulatory Decision-Making: Current Status and Emerging Priorities. Environ Mol Mutagen [Internet]. 2020 Jan 1 [cited 2022 Jul 14];61(1):66–83. Available from: https://pubmed.ncbi.nlm.nih.gov/31794061/

25. More S, Bampidis V, Benford D, Bragard C, Halldorsson T, Hougaard Bennekou S, et al. Statement on the derivation of Health-Based Guidance Values (HBGVs) for regulated products that are also nutrients. EFSA Journal. 2021 Mar;19(3).

26. White PA, Long AS, Johnson GE. Quantitative Interpretation of Genetic Toxicity Dose-Response Data for Risk Assessment and Regulatory Decision-Making: Current Status and Emerging Priorities. Environ Mol Mutagen. 2020 Jan 19;61(1):66–83.

27. Steiblen G, Benthem J van, Johnson G. Strategies in genotoxicology: Acceptance of innovative scientific methods in a regulatory context and from an industrial perspective. Mutation Research/Genetic Toxicology and Environmental Mutagenesis. 2020 May;853:503171.

28. Boisvert L, Derr R, Osterlund T, Hendriks G, Brandsma I. Quantitative Interpretation of ToxTracker Dose Response Data for Potency Comparisons and Mode-of-Action Determination. Environ Mol Mutagen. 2023 Jan 16;

29. Marc A. Beal, Marc Audebert, Tara Barton-Maclaren, Hannah Battaion, Jeffrey C. Bemis, Xuefei Cao, et al. Application of Quantitative in vitro to in vivo Extrapolation (IVIVE) Modeling to in vitro Genotoxicity Data Provides Conservative Estimates of in vivo Genotoxicity.

30. Boisvert L, Derr R, Osterlund T, Hendriks G, Brandsma I. Quantitative Interpretation of ToxTracker Dose Response Data for Potency Comparisons and Mode-of-Action Determination Environmental and Molecular Mutagenesis.

31. Kirkland D, Reeve L, Gatehouse D, Vanparys P. A core in vitro genotoxicity battery comprising the Ames test plus the in vitro micronucleus test is sufficient to detect rodent carcinogens and in vivo genotoxins. Mutation Research/Genetic Toxicology and Environmental Mutagenesis. 2011 Mar;721(1):27–73.

32. Madia F, Kirkland D, Morita T, White P, Asturiol D, Corvi R. EURL ECVAM Genotoxicity and Carcinogenicity Database of Substances Eliciting Negative Results in the Ames Test: Construction of the Database. Mutation Research/Genetic Toxicology and Environmental Mutagenesis. 2020 Jun;854–855:503199.

33. Morita T, Uno Y, Honma M, Kojima H, Hayashi M, Tice RR, et al. The JaCVAM international validation study on the in vivo comet assay: Selection of test chemicals. Mutation Research/Genetic Toxicology and Environmental Mutagenesis. 2015 Jul;786–788:14–44.

34. R Core Team. A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. [Internet]. 2022 [cited 2022 Oct 10]. Available from: https://www.R-project.org/

35. Whysner J, Ross PM, Conaway CC, Verna LK, Williams GM. Evaluation of possible genotoxic mechanisms for acrylonitrile tumorigenicity. Regul Toxicol Pharmacol [Internet]. 1998 [cited 2022 Jul 20];27(3):217–39. Available from: https://pubmed.ncbi.nlm.nih.gov/9693074/

36. Whysner J, Steward RE, Chen D, Conaway CC, Verna LK, Richie JP, et al. Formation of 8-oxodeoxyguanosine in brain DNA of rats exposed to acrylonitrile. Arch Toxicol [Internet]. 1998 [cited 2022 Jul 20];72(7):429–38. Available from: https://pubmed.ncbi.nlm.nih.gov/9708882/

37. Eastmond DA. Benzene-induced genotoxicity: a different perspective. J Toxicol Environ Health A [Internet]. 2000

[cited 2022 Jul 20];61(5–6):353–6. Available from: https://pubmed.ncbi.nlm.nih.gov/11086939/

38. Whysner J. Benzene-induced genotoxicity. J Toxicol Environ Health A. 2000;61(5–6):347–51.

39. IARC. Benzene. Benzene [Internet]. 2018 [cited 2022 Jul 20]; Available from: https://pubmed.ncbi.nlm.nih.gov/31769947/

40. Fahmy MA, Aly FAE. In vivo and In vitro Studies on the Genotoxicity of Cadmium Chloride in Mice. JOURNAL OF APPLIED TOXICOLOGY J Appl Toxicol. 2000;20:231–8.

41. Westphal GA, Bünger J, Schulz TG, Müller MM, Hallier E. Mutagenicity of N -nitrosodiethylamine in the Ames test with S. typhimurium TA1535 is due to volatile metabolites and is not dependent on cytochrome P4502E1 induction. Arch Toxicol. 2000 Dec 11;74(10):638–41.

42. Liu X, Wu J, Shi W, Shi W, Liu H, Wu X. Lead Induces Genotoxicity via Oxidative Stress and Promoter Methylation of DNA Repair Genes in Human Lymphoblastoid TK6 Cells. Med Sci Monit [Internet]. 2018 Jun 22 [cited 2022 Jul 21];24:4295. Available from: /pmc/articles/PMC6045917/

43. NTP. NTP website.

44. Allemang A, de Abrew KN, Shan YK, Krailler JM, Pfuhler S. A comparison of classical and 21st century genotoxicity tools: A proof of concept study of 18 chemicals comparing in vitro micronucleus, ToxTracker and genomics-based methods (TGx-DDI, whole genome clustering and connectivity mapping). Environ Mol Mutagen. 2021 Feb 1;62(2):92–107.

45. Cho SH, Guengerich FP. In vivo roles of conjugation with glutathione and O6-alkylguanine DNA-alkyltransferase in the mutagenicity of the bis-electrophiles 1,2-dibromoethane and 1,2,3,4-diepoxybutane in mice. Chem Res Toxicol [Internet]. 2013 Nov 18 [cited 2022 Jul 22];26(11):1765–74. Available from: https://pubmed.ncbi.nlm.nih.gov/24191644/

46. Barilli A, Atzeri C, Bassanetti I, Ingoglia F, Dall'Asta V, Bussolati O, et al. Oxidative Stress Induced by Copper and Iron Complexes with 8-Hydroxyquinoline Derivatives Causes Paraptotic Death of HeLa Cancer Cells. Mol Pharm. 2014 Apr 7;11(4):1151–63.

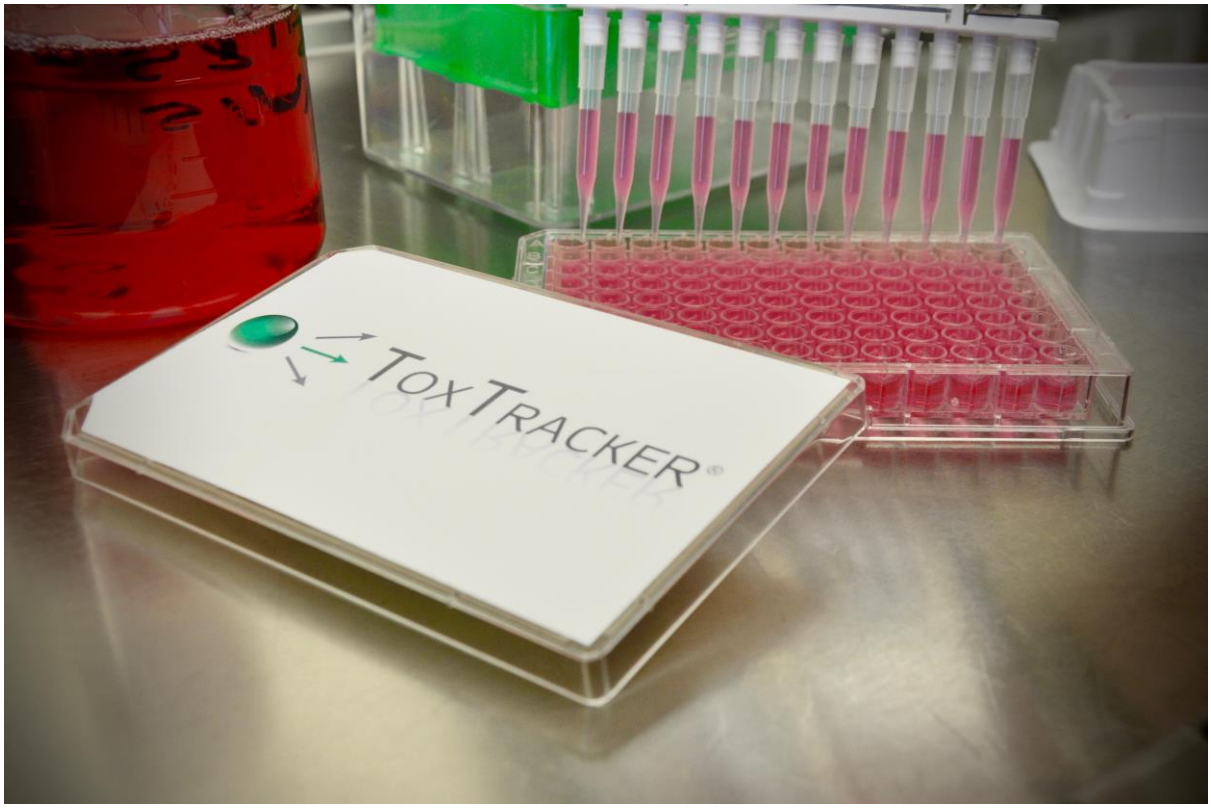
47. Hernández LG, van Steeg H, Luijten M, van Benthem J. Mechanisms of non-genotoxic carcinogens and importance of a weight of evidence approach. Mutation Research/Reviews in Mutation Research. 2009 Sep 1;682(2–3):94–109.

48. Fowler P, Smith R, Smith K, Young J, Jeffrey L, Kirkland D, et al. Reduction of misleading (“false”) positive results in mammalian cell genotoxicity assays. II. Importance of accurate toxicity measurement. Mutat Res [Internet]. 2012 Aug 30 [cited 2022 Jul 22];747(1):104–17. Available from: https://pubmed.ncbi.nlm.nih.gov/22595317/

49. Woźniak K, Blasiak J. In vitro genotoxicity of lead acetate: induction of single and double DNA strand breaks and DNA-protein cross-links. Mutat Res [Internet]. 2003 Mar 3 [cited 2022 Jul 22];535(2):127–39. Available from: https://pubmed.ncbi.nlm.nih.gov/12581530/

Annex 1: Full ToxTracker protocol

ToxTracker assay protocol



Authors: Remco Derr
Giel Hendriks

Version: Final validation protocol (v2.3)
Date: 26th of October 2018

Table of content.

1. Background

The ToxTracker assay is a panel of GFP-based mouse embryonic stem (mES) reporter cell lines that can be used to identify the biological reactivity and potential carcinogenic properties of newly developed chemicals in a single test (Hendriks, 2012; Hendriks, 2016). ToxTracker is a mammalian stem cell-based assay that monitors activation of specific cellular signalling pathways for detection of the biological reactivity of compounds (Hendriks, 2013). In contrast to the cancer-derived cell lines that are currently used for *in vitro* genotoxicity testing, stem cells are genetically stable and proficient in all cellular pathways required for accurate detection of potentially carcinogenic properties of compounds. Extensive whole-genome transcription profiling has led to identification of a panel of biomarker genes that are preferentially activated upon exposure to different classes of carcinogens and toxicants (Hendriks, 2011). To allow easy assessment of the activation status of these biomarker genes, we have generated green fluorescent (GFP) mES reporter cell lines. These reporters were created using artificial chromosomes that contain the complete biomarker gene including promoter and regulatory elements ensuring physiological regulation of the GFP reporters following transfection into stem cells.

ToxTracker consists of a panel of six different mES GFP reporter cell lines representing four distinct biological responses that are associated with carcinogenesis, i.e. general cellular stress, DNA damage, oxidative stress and the unfolded protein response (Table 1).

Table 1: Specificity of the ToxTracker reporters.

Biological damage	Cellular pathway	Biomarker gene	MOA
DNA damage	ATR/Chk1 DNA damage signaling	Bscl2	Mutagenic DNA lesions
	NF-kB signaling	Rtkn	DNA double-strand breaks
Oxidative stress	Nrf2 antioxidant response	Srxn1	ROS production
	Nrf2-independent	Blvrb	ROS production
Protein damage	Unfolded protein response	Ddit3	Protein damage
Cellular stress	p53 signaling	Btg2	Cytotoxicity

2. Materials and equipment

- 6 ToxTracker reporter cell lines
- Wild type mES cells (B4418)
- Cell culture 96-wells plates
- Round bottom 96-wells plates (for compound dilutions)
- Sterile 0.1% gelatin in water solution (Sigma-Aldrich, G1890-100G)

- mES cell culture medium (see annex I)
- 0.05% trypsin-EDTA solution (Gibco 25300096)
- Sterile PBS (Gibco 14190094)
- 2% foetal bovine serum (FBS) in PBS
- Aroclor-1254 induced male Sprague Dawley rat liver S9, in 0.15 M KCl (Moltox 11-101.5)
- NADPH Regensys™ A solution (Moltox 60-200.5)
- NADPH Regensys™ B (Moltox 60-201.5L)
- DMSO
- Multichannel liquid reservoirs (12 well)
- 50 ml liquid reservoirs

- CO_2 incubator (37℃, 5% CO_2)
- Laminar flow cabinet
- Centrifuge with rotor for 15 ml tubes
- Water bath
- Multichannel pipet (20-200 µl)
- Multichannel pipet (2-20 µl)
- Pipets + tips
- Cell counter

3. Preparation of cell culture plates (day 1)

All 96-wells plates should be coated with gelatin before seeding of the ToxTracker reporter cell lines

- Add 50 µl 0.1% gelatin solution to each well of a 96-wells plate using a multichannel pipet
- Incubate for at least 5 min at RT (up to few hours)
- Start preparation of the cell lines

Instructions on cell viability
Only start the dose finding if the wild type mES cells are growing properly. The condition of the cell culture can be assessed by checking the morphology of the cells and by cell count. Two days after seeding the cells, the culture dish should be 70-90% confluent. The stem cells should be undifferentiated meaning that they , have a uniform morphology, it's difficult to identify individual cells and when the dishes get confluent often grow in dense clusters.
Proper cell growth is also addressed by cell count. When $5x10^6$ cells (p90) or $2x10^6$ cells (p60) were seeded on day 1, a healthy culture should result in $20\text{-}25x10^6$ (p90) or $8\text{-}12x10^6$ (p60) cells on day 3. If these cell numbers are not reached, the dose finding should be postponed. In that case, cells should be tripsinised and passed to fresh culture dishes according to the standard ToxTracker cell culture protocol.

4. Seeding wild-type mESC for dose-finding (day 1)

- Warm mESC medium in a 37°C water bath
- Aspirate medium from the mESC plates
- Wash cells twice with 3.5 ml (60 mm dishes) or 7 ml (90 mm dishes) PBS
- Add 0.5 ml (60 mm dishes) or 1 ml (90 mm dishes) trypsin-EDTA solution
- Incubate 5 min at room temperature (until the cells detach)
- Prepare a 15 ml tube with 2 ml (60 mm dishes) or 4 ml (90 mm dishes) warm mESC medium
- Resuspend the wild type mES cells in the trypsin-EDTA solution (DO NOT ADD MEDIUM)
- Transfer the cell suspension to the 15 ml tube
- Mix thoroughly
- Determine cell concentration
- Calculate the number of cells that is required for the assay (Table 2), 1 row per compound is required. Add +/-10% extra cells/volume for pipetting errors.
- Always include 2 extra rows for the control compounds (Cisplatin (5µM -S9 and +S9) & Aflatoxin B1 (5µM -S9 and +S9). See table 3 for a sample of plate lay-out.
- Include always 1 extra row of each cell line in a separate plate to count on the day of treatment for RPD / RICC calculations!
- Discard gelatin solution completely from the plate (no washing of the plate).
- Add 200 µl wild type mES cell suspension to each well of the 96-wells plate. See sample plate layout below (Table 3).
- Place cells in a cell culture incubator at 37°C, 5% CO2

Table 2: Cell numbers required for dose-finding

No. of rows	Number of cells	Volume mESC medium
1	$0.48x10^6$	2.4 ml
2	$0.96x10^6$	4.8 ml
3	$1.44x10^6$	7.2 ml
4	$1.92x10^6$	9.6 ml
5	$2.40x10^6$	12.0 ml
6	$2.88x10^6$	14.4 ml
7	$3.36x10^6$	16.8 ml
8	$3.84x10^6$	19.2 ml
9	$4.32x10^6$	21.6 ml
10	$4.80x10^6$	24.0 ml

Table 3: Sample plate design for dose-finding in wild type mESCs. Compound concentrations in µg/ml.

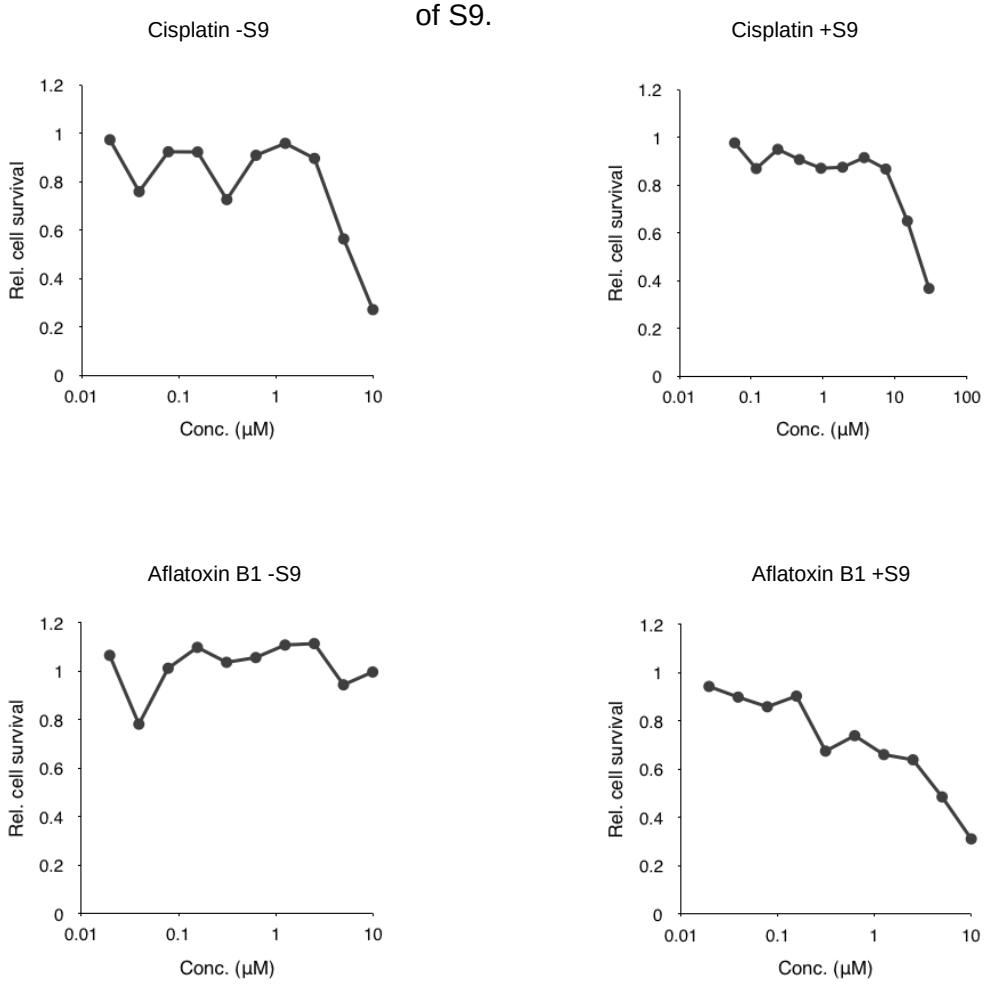
	1	2	3	4	5	6	7	8	9	10	11	12	
A	0	0,001	0,004	0,02	0,06	0,2	1	3,9	15,6	62,5	250	1000	Compound 1
B	0	0,001	0,004	0,02	0,06	0,2	1	3,9	15,6	62,5	250	1000	Compound 2
C	0	0,001	0,004	0,02	0,06	0,2	1	3,9	15,6	62,5	250	1000	Compound 3
D	0	0,001	0,004	0,02	0,06	0,2	1	3,9	15,6	62,5	250	1000	Compound 4
E	0	0,001	0,004	0,02	0,06	0,2	1	3,9	15,6	62,5	250	1000	Compound 5
F	0	0,001	0,004	0,02	0,06	0,2	1	3,9	15,6	62,5	250	1000	Compound 6
G	0	0,001	0,004	0,02	0,06	0,2	1	3,9	15,6	62,5	250	1000	Compound 7
H	0	0,001	0,004	0,02	0,06	0,2	1	3,9	15,6	62,5	250	1000	Compound 8

Table 4: Sample plate design for the dose-finding controls. Compound concentrations in µM.

	1	2	3	4	5	6	7	8	9	10	11	12	
A	0	0,01	0,02	0,04	0,08	0,2	0,3	0,6	1,3	2,5	5	10	CisPt -S9
B	0	0,01	0,02	0,04	0,08	0,2	0,3	0,6	1,3	2,5	5	10	AFB1 -S9
C	0	0,01	0,02	0,04	0,08	0,2	0,3	0,6	1,3	2,5	5	10	CisPt +S9
D	0	0,01	0,02	0,04	0,08	0,2	0,3	0,6	1,3	2,5	5	10	AFB1 +S9
E													
F													
G													
H													

Control plate

Figure 1: Example dose response for the control compounds CisPt and AFB1 in absence and presence of S9.



5. Preparation of compound dilution series (day 1)

Every chemical/substance that is tested in the ToxTracker assay is first analysed for cytotoxicity in a broad dose range finding. For the dose range finding, a maximum concentration of 1 mg/ml or 1 µl/ml is used. In case concentrations are limited by solubility or the occurrence of precipitation in the culture medium, the maximum soluble concentration will be used in the assay (see below). 11 different concentrations for the test substance will be tested, starting at the maximum concentration and ten consecutive 4-fold dilutions. Later in the ToxTracker assay, compounds will be tested in 2-fold dilution series. Therefore, already during compound dilution preparations for the dose finding, 22 serial dilutions in 2-fold dilution steps will be made. From these 22 dilutions series, the appropriate dilutes will be used for the dose finding. The 2-fold dilution series will be prepared in 96-wells round bottom plates for easy handling of the dilutions using a multi channel pipet. Please find the overview of the compound concentration range and plate layout below.

Instructions on compound solubility
Solubility is initially assessed by eye when the stock solutions are prepared. In case a compound does not dissolve completely in the proposed solvent at room temperature, the solution can be warmed at 37 ℃ or placed in a sonication bath for some time. When the compounds still does not dissolve at all in the proposed solvent, please contact Els for instructions. When a compound partly dissolves, continue with preparations of the compound dilution series as indicated below, even if you see some precipitation at higher concentrations. Make sure you create a homogeneous suspension before you prepare the dilution range. In case of partial soluble, it is highly recommend to prepare the dilution series in 1.5 ml Eppendorf tubes in stead of a multi-well plate.
The maximum test concentration in the dose range finding is 1 mg/ml. Expose the cells to the compound dilutions. Solubility of the compounds will be assessed in the cell culture medium **at the end of the 24 h. exposure**. Precipitation in the cell cultures should be observed under a microscope. Define the maximum soluble concentration at the end of the dose range finding. The top doses for the ToxTracker analysis should be based on cytotoxicity as described below, but is limited by the maximum soluble concentrations in cell culture medium after 24 h. incubation at 37 ℃.

Table 5: Compound concentration range that will be prepared for the dose finding and ToxTracker assay. Concentrations that will be applied in the dose finding are indicated in red.

Compound dilution	Prepared stock dilution (µg/ml)	Final concentration in well
1	0,0476837158203125	0,000476837158203125
2	**0,095367431640625**	**0,00095367431640625**
3	0,19073486328125	0,0019073486328125
4	**0,3814697265625**	**0,003814697265625**
5	0,762939453125	0,00762939453125
6	**1,52587890625**	**0,0152587890625**
7	3,0517578125	0,030517578125
8	**6,103515625**	**0,06103515625**
9	12,20703125	0,1220703125
10	**24,4140625**	**0,244140625**
11	48,828125	0,48828125
12	**97,65625**	**0,9765625**
13	195,3125	1,953125
14	**390,625**	**3,90625**
15	781,25	7,8125
16	**1.562,5**	**15,625**
17	3.125	31,25
18	**6.250**	**62,5**
19	12.500	125
20	**25.000**	**250**
21	50.000	500
22	**100.000**	**1000**

Table 6: Plate layout for preparations of the 2-fold dilution series. 22 serial dilutions are prepared. Dilutions that will be applied in the dose range finding are indicated in red.

Dilution plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	1	2	3	4	5	6	7	8	9	10	11
B	0	1	2	3	4	5	6	7	8	9	10	11
C	0	1	2	3	4	5	6	7	8	9	10	11
D	0	1	2	3	4	5	6	7	8	9	10	11
E	0	1	2	3	4	5	6	7	8	9	10	11
F	0	1	2	3	4	5	6	7	8	9	10	11
G	0	1	2	3	4	5	6	7	8	9	10	11
H	0	1	2	3	4	5	6	7	8	9	10	11

Dilution plate 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	12	13	14	15	16	17	18	19	20	21	22	
B	12	13	14	15	16	17	18	19	20	21	22	
C	12	13	14	15	16	17	18	19	20	21	22	
D	12	13	14	15	16	17	18	19	20	21	22	
E	12	13	14	15	16	17	18	19	20	21	22	
F	12	13	14	15	16	17	18	19	20	21	22	
G	12	13	14	15	16	17	18	19	20	21	22	
H	12	13	14	15	16	17	18	19	20	21	22	

- Prepare for each compound a 100 mg/ml solution in DMSO or water according to the MSDS sheet or instructions from the Compound Selection Team.
- In case of liquid compounds, prepare a 100 µl/ml stock solution. The compound is diluted in DMSO unless instructed otherwise.
- Dilute the compound in 22 consecutive 2-fold dilutions in the appropriate solvent
- Have at least 125 µl for each dilution available
- Dilutions can be prepared in 96-wells round bottom plates or multi-tube strips as shown in table 6
- For convenience, dilution series can be prepared a number of days before the dose range finding and stored at -20℃ for future testing.

6. Exposure of the wild-type mESC for dose-finding (day 2)

24 h after seeding of the wild type mES cells in the 96-well plates, fresh ES cell medium containing the diluted chemicals is added to the cells. For all compounds 11 4-fold dilutions are tested. The maximum tested concentration that will be tested in the dose finding is 1 mg/ml. Also a vehicle control is included. The reference compounds cisplatin and aflatoxin B1 are included as positive controls (maximum tested concentration 5 µM) **The dose range finding is performed in absence and presence of S9.**

Exposure in absence of S9

- Warm mESC medium in a 37°C water bath
- Prepare mESc medium with the dilution series of the test compound (198 µl medium + 2 µl compound). 11 consecutive 4-fold dilutions (see tables 5 and 6).
- Aspirate medium from the reporter cell 96-wells plates
- Add 200 µl of the compound dilution in medium to the cells. A sample plate design can be found in Tables 3 and 4.
- Store plates for 24 h in a cell culture incubator at 37°C, 5% CO2
- Important: Perform a cell count in the row of cells that was seeded separately to find the cell concentration at the moment of treatment for calculation of RPD and RICC.
- Warm up the Trypsin-EDTA solution in water bath
- Aspirate cell culture medium
- Wash cells twice with 200 µl PBS
- Completely aspirate PBS and add 40 µl trypsin-EDTA solution
- Incubate for 5 min at room temperature
- Resuspend cells in trypsin-EDTA solution (DO NOT ADD MEDIUM)
- Add 110 µl of cold PBS supplemented with 2% FBS
- Perform a cell count by flow cytometry. Alternatively, manual or automated cell count (e.g. Coulter counter) can be used.

Exposure in presence of S9

- Warm mESC medium in a 37°C water bath
- Prepare mESC medium with the dilution series of the test compound (193 µl medium + 2 µl compound). 11 consecutive 4-fold dilutions (see tables 5 and 6).
- Prepare a 10% S9 rat liver (aroclor-1254 induced rats) solution with the RegenSysA/B cofactor solutions (according to the Moltox manufacturers protocols)
- Add 5 µl of the 10% S9 solution to every compound dilution
- Aspirate medium from the reporter cell 96-wells plates
- Add 200 µl of the compound dilutions in medium containing 0.25% S9 to the cells. A sample plate design can be found in Tables 3 and 4.
- Store plates for 24 h in a cell culture incubator at 37°C, 5% CO2

7. Analysis of dose-finding by Flow Cytometry (day 3)

Cell concentrations in each well are determined after 24h exposure in absence or presence of S9. Cell count in the wells is used to estimate the relative cell survival after exposure.

- Warm up the Trypsin-EDTA solution in water bath
- **CHECK CELL CULTURES FOR COMPOUND PRECIPITATION**
- Aspirate cell culture medium
- Wash cells twice with 200 µl PBS
- Completely aspirate PBS and add 40 µl trypsin-EDTA solution
- Incubate for 5 min at room temperature

- Resuspend cells in trypsin-EDTA solution (DO NOT ADD MEDIUM)
- Add 110 µl of cold PBS supplemented with 2% FBS
- Analyse samples by flow cytometry
- IMPORTANT: check samples for GFP induction of selected concentrations for autofluorescence of the compound. If an increase of 2-fold or more is observed, seed wild-type mESC during seeding of ToxTracker reporter cell lines for **both -S9 and +S9** treatments to correct for autofluorescence of compound.

Selection of the compound concentration for ToxTracker analysis.

Selection of the maximum concentration that will be applied in the ToxTracker assay will be determined based on cell count. The cell concentration after compound exposure is divided by the cell concentration of the vehicle control exposed cells. Cytotoxicity based on RPD will be calculated for data analysis, but will not be used for selection of the maximum tested concentration. The top concentration that is selected for ToxTracker induces 50-75% cytotoxicity. Concentration selection for -S9 and +S9 should be equal. Only in cases where a more >4-fold difference in top concentration is observed, you can chose a different top concentration for -S9 and +S9 treatment. Concentrations that induce >75% cytotoxicity, corresponding to <0.25 relative cell survival in the ToxTracker results spreadsheet, should not be included in this study. In the case that a tested concentration gives <50% cytotoxicity and the following testing concentration (4-fold higher) induces >75% cytotoxicity, the non-tested intermediate concentration should be selected as top concentration for the ToxTracker analysis.
In case no cytotoxicity is observed, the maximum concentration of 1 mg/ml is applied in ToxTracker. Selection of the top concentration for the ToxTracker analysis can also be limited by solubility of the compound (see instructions above).

8. Preparation of cell culture plates (day 3)

All 96-wells plates should be coated with gelatin before seeding of the ToxTracker reporter cell lines

- Add 50 µl 0.1% gelatin solution to each well of a 96-wells plate using a multichannel pipet
- Incubate for at least 5 min at room temperature (up to few hours)
- Start preparation of the ToxTracker reporter cell lines

Instructions on cell viability
Only start the ToxTracker assay if the reporter cells are growing properly. The condition of the cell culture can be assessed by checking the morphology of the cells and by cell count. Two days after seeding the reporter cells, the culture dish should be 70-90% confluent. The stem cells should be undifferentiated meaning that they have a uniform morphology, it's difficult to identify individual cells and when the dishes get confluent often grow in dense clusters.
Proper cell growth is also addressed by cell count. When $5x10^6$ cells (p90) or $2x10^6$ cells (p60) were seeded on day 1, a healthy culture should result in $20\text{-}25x10^6$ (p90) or $8\text{-}12x10^6$ (p60) cells on day 3. If these cell numbers are not reached, the dose finding should be postponed. Cells should be tripsinised and passed to fresh culture dishes according to the standard ToxTracker cell culture protocol.

9. Seeding ToxTracker reporter cell lines (day 3)

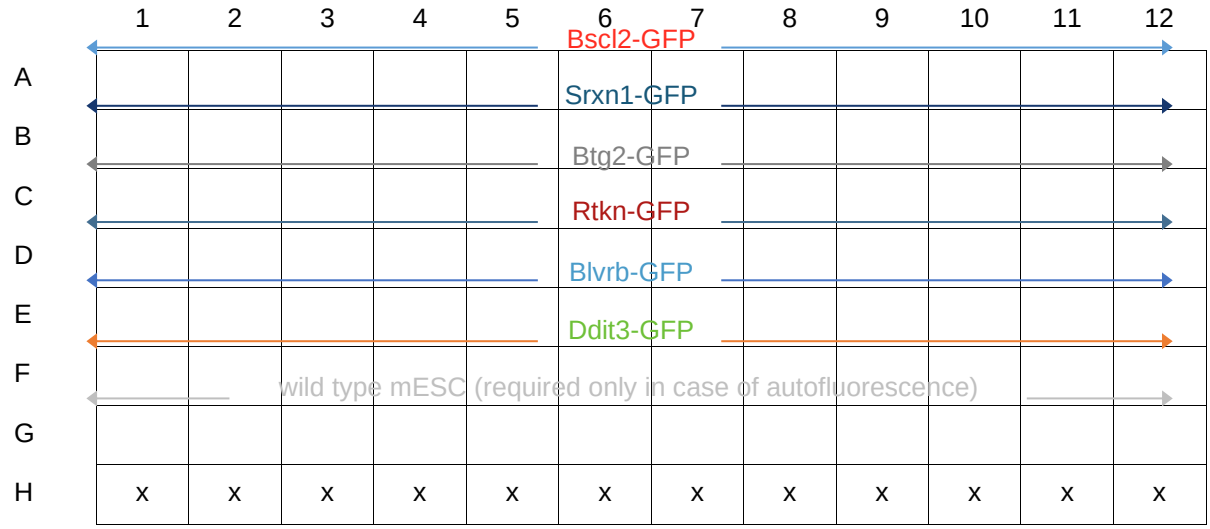
- Warm mESC medium in a 37°C water bath
- Aspirate medium from the mESC plates
- Wash cells twice with 3.5 ml (60 mm dishes) or 7 ml (90 mm dishes) PBS
- Add 0.5 ml (60 mm dishes) or 1 ml (90 mm dishes) trypsin-EDTA solution
- Incubate 5 min at room temperature (until the cells detach)
- Prepare for each cell line a 15 ml tube with 2 ml (60 mm dishes) or 4 ml (90 mm dishes) warm mESC medium
- Resuspend the mES cells in the trypsin-EDTA solution (DO NOT ADD MEDIUM)
- Transfer the cell suspension to the 15 ml tube
- Mix thoroughly
- Determine cell concentration
- Calculate the number of cells that is required for the assay (Table 4). Add 10% extra cells/volume to ensure sufficient cell suspension for seeding.
- Always include an extra control plate for the four reference compounds.

- Include a second extra plate to count cell concentration at the day of treatment for RPD and RICC calculations.
- Discard gelatin solution completely from the plate (no washing of the plate).
- For each reporter cell line, add 200 µl cell suspension to each well of the 96-wells plate. Seed each row of the plate with a different reporter cell line. See sample plate layout below (Table 8).
- DO NOT ADD G418 to the plates
- Place cells in a cell culture incubator at 37°C, 5% CO2

Table 7: Cell numbers required for ToxTracker analysis

No. of plates	Number of cells	Volume mESC medium
1	$0.48x10^6$	2.4 ml
2	$0.96x10^6$	4.8 ml
3	$1.44x10^6$	7.2 ml
4	$1.92x10^6$	9.6 ml
5	$2.40x10^6$	12.0 ml
6	$2.88x10^6$	14.4 ml
7	$3.36x10^6$	16.8 ml
8	$3.84x10^6$	19.2 ml
9	$4.32x10^6$	21.6 ml
10	$4.80x10^6$	24.0 ml

Table 8: Sample plate design for the ToxTracker assay



10. Exposure of the ToxTracker reporter cell lines (day 4)

24h after seeding the cells in the 96-well plates, fresh ES cell medium containing the diluted chemicals is added to the cells. The compound dilutions were already prepared during the dose range finder. For each tested compound, five concentrations are tested in 2-fold dilutions. The highest compound concentration will induce significant cytotoxicity (50-75% cell death), or will be the maximum soluble compound concentration or 1 mg/ml (in case of no/low cytotoxicity). Solubility is determined during the dose range finding as described above. Positive reference treatments with cisplatin (DNA damage), diethyl maleate (oxidative stress), tunicamycin (unfolded protein response) and aflatoxin B1 (S9 metabolism) are included in all experiments.

Exposure in absence of S9

- Warm mESC medium in a 37°C water bath
- Prepare mESC medium with the dilution series of the test compound (1.5 ml medium + 15 µl compound). Five consecutive 2-fold dilutions.
- For each experiment, a control plate should be prepared with the positive controls cisplatin, diethyl maleate, tunicamycin and Aflatoxin B1. For the control compounds, two concentrations are included. See table 9 for a standard control plate layout.
- Aspirate medium from the reporter cell 96-wells plates
- Add 200 µl of the compound dilutions to the reporter cell lines. A sample plate design can be found in Table 10.
- Store plates in a cell culture incubator at 37°C, 5% CO2
- Perform a cell count on the extra plate that was seeded on day 3 for the calculation of RPD and RICC. Cell count can be performed manually, in an automated cell counter or by using a flow cytometer. For flow cytometry-based cell count, counting beads can be added to the cell suspension for a reliable absolute cell number calculation.
- Warm up the Trypsin-EDTA solution in water bath
- Aspirate cell culture medium
- Wash cells twice with 200 µl PBS
- Completely aspirate PBS and add 40 µl trypsin-EDTA solution
- Incubate for 5 min at room temperature
- Resuspend cells in trypsin-EDTA solution (DO NOT ADD MEDIUM)
- Add 110 µl of cold PBS supplemented with 2% FBS
- Analyse samples by flow cytometry

Table 9: Sample plate design for the ToxTracker control plate.

	1	2	3	4	5	6	7	8	9	10	11	12
Bscl2-GFP	0	2,5	5	0	125	250	0	2	4	0	2,5	5
Srxn1-GFP	0	2,5	5	0	125	250	0	2	4	0	2,5	5
Btg2-GFP	0	2,5	5	0	125	250	0	2	4	0	2,5	5
Rtkn-GFP	0	2,5	5	0	125	250	0	2	4	0	2,5	5
Blvrb-GFP	0	2,5	5	0	125	250	0	2	4	0	2,5	5
Ddit3-GFP	0	2,5	5	0	125	250	0	2	4	0	2,5	5
	x	x	x	x	x	x	x	x	x	x	x	x
	x	x	x	x	x	x	x	x	x	x	x	x

CisPt DEM Tunica AFB1 +S9

Table 10: Sample plate design for the compound testing in the ToxTracker assay

	1	2	3	4	5	6	7	8	9	10	11	12
Bscl2-GFP	0	0,625	1,25	2,5	5	10	0	6,25	12,5	25	50	100
Srxn1-GFP	0	0,625	1,25	2,5	5	10	0	6,25	12,5	25	50	100
Btg2-GFP	0	0,625	1,25	2,5	5	10	0	6,25	12,5	25	50	100
Rtkn-GFP	0	0,625	1,25	2,5	5	10	0	6,25	12,5	25	50	100
Blvrb-GFP	0	0,625	1,25	2,5	5	10	0	6,25	12,5	25	50	100
Ddit3-GFP	0	0,625	1,25	2,5	5	10	0	6,25	12,5	25	50	100
mESC (when AF)	0	0,625	1,25	2,5	5	10	0	6,25	12,5	25	50	100
	x	x	x	x	x	x	x	x	x	x	x	x

Compound 1 Compound 2

Exposure in presence of S9

- Warm mESC medium in a 37°C water bath
- Prepare mESC medium with the dilution series of the test compound (1.5 ml medium + **15.4 µl** compound). Five consecutive 2-fold dilutions
- Prepare a 10% S9 rat liver (aroclor-1254 induced rats) solution with the RegenSysA/B cofactor solution (according to the Moltox manufacturers protocols)

- Add 37.5 µl to each medium + compound mix (1.5 ml + 15.4 µl mix) to obtain a 0.25% S9 concentration in the medium + compound mix solution.
- Aspirate medium from the reporter cell 96-wells plates
- Add 200 µl of the compound dilutions with S9 to the reporter cell lines. A sample plate design can be found in Table 10 (the positive control Aflatoxin B1 +S9 is included on the control plate, Table 9).
- Store plates for 24 h in a cell culture incubator at 37°C, 5% CO2

11. Analysis of ToxTracker reporter induction (day 5)

Induction of the GFP reporters is determined after 24 h exposure using a flow cytometer. Only GFP expression in intact single cells is determined. Mean GFP fluorescence in each well is measured. During GFP detection, also cell concentration in each well is determined and used for cytotoxicity assessment.

ToxTracker analyses can be performed on various flow cytometers with 96-well plate capabilities. Proper settings for the flow cytometer should be verified before running ToxTracker by testing the control compounds in the ToxTracker cell lines. Important parameters when setting the proper instrument settings are:

- separation of intact cells from broken cells and debris
- appropriate fluorescence levels of the untreated control cells
- Detection of fluorescence induction in treated reporter cells

Guidance on setting the appropriate cell gates and fluorescence levels for the BD FacsCanto, Millipore Guava and Miltenyi MacsQuant can be found in Annex III.

- Warm up the Trypsin-EDTA solution in a 37ºC water bath
- Aspirate cell culture medium
- Wash twice with 200 µl PBS
- Completely remove PBS and add 40 µl of trypsin-EDTA solution
- Incubate for 5 min at room temperature
- Resuspend cells in trypsin-EDTA solution (DO NOT ADD mESC MEDIUM)
- Add 110 µl cold 2% FBS in PBS solution
- Analyse samples by flow cytometry.
- Determine mean GFP fluorescence in 5000 intact mES cells as well as the cell concentration in each well. Alternatively on the BD FacsCanto flow cytometers, a fixed volume (**30 µl**) of cell suspension can be analysed to measure GFP induction and to estimate the cell concentration in the wells.
- Calculate relative GFP induction levels in exposed reporter cells compared to the vehicle control exposed cells.
- Calculate relative cell survival in exposed reporter cells compared to the vehicle control exposed cells.

12. Quality controls for the ToxTracker assay

To ensure proper performance of the ToxTracker assay, various quality control checks are included in the protocol. In case the quality limits are not met, the results from this ToxTracker test should be discarded and a repeat experiment should be conducted.

The quality of the cells that are applied in the ToxTracker assay is assessed from **cell count** of vehicle control treated samples in the assay. At the start of the ToxTracker analysis, 40,000 cells are seeded per well of the 96-wells plate. After two days when the ToxTracker reporter cell lines are analysed for GFP expression, the **minimum cell concentration** per well should be $4x10^5$ cells/ml for all the cell lines (-20% for the Srxn1-GFP reporter because of slower cell growth).

GFP induction and cytotoxicity levels of the positive controls validate the overall quality of the ToxTracker assay. Treatment with Cisplatin (DNA damage), Diethyl maleate (oxidative stress), Tunicamycin (protein unfolding) and Aflatoxin B1 (S9 metabolism) are standard controls for theToxTracker assay and should be included in every experiment. For each control, two concentrations are included in the control plates as indicated in Table 9. Control compounds should results in a **minimal GFP induction levels** in the relevant ToxTracker reporter cell lines as indicated in Table 11. In case the GFP induction levels are below these thresholds, all test results from this experiment should be discarded. Cytotoxicity for Cisplatin at 5 µM is 50% (+/-20%). For Diethyl maleate cytotoxicity levels should be around 50% (+/-30%) at 250 µM. Tunicamycin induces a cytotoxicity level of 50% (+/-20%) at a concentration of 4 µg/ml. For Aflatoxin B1 (5 µM) cytotoxicity levels should be around 50% (+/-20%) in presence of S9. Discard the experiment if those levels of cytotoxicity are not reached. If the quality of the cells was not acceptable, prepare fresh control compound dilutions.

Table 11: Minimum induction levels for the ToxTracker reporter cell lines by the relevant control compound.

Cell line	Compound treatment	Min. relative GFP induction level
Bscl2-GFP	Cisplatin/Aflatoxin B1+S9	2
Srxn1-GFP	DEM	8
Btg2-GFP	Cisplatin/Aflatoxin B1+S9	2
Rtkn-GFP	Cisplatin/Aflatoxin B1+S9	3
Blvrb-GFP	DEM	5
Ddit3-GFP	Tunicamycin	4

Autofluorescence by the test compound may interfere with the measurement of GFP reporter induction. Therefore, GFP levels are measured in wild-type mES during the dose range finding. If a relative induction of 2 or more is found at concentrations that will be

applied during the ToxTracker assay, wild-type mES cells should be included in the ToxTracker assay to correct afterwards for the level of autofluorescence caused by the compound. In case of autofluorescence, the green fluorescence signals are subtracted from the GFP reporter fluorescence levels in the ToxTracker assay. After green fluorescence correction, relative inductions of the GFP reporters in exposed cells is calculated as described above in the protocol. In case autofluorescence is observed for a compound, wild type mES cells should be included in all tests -S9 and +S9 for this compound to perform fluorescence correction.

Additional checks:

- Changes of batches of material should be monitored with regard to their influence on principal endpoints in use in a study.
- Check cells each day for confluence, adherence, cell morphology and contamination.
- The impact of variation of cell proliferation and cell differentiation should be monitored and documented.
- Perform checks to control functionality of FACS.
- Solubility of the test compounds should be verified during the dose range finding, prior to the ToxTracker analysis.
- Changes in pH of the cell culture medium by the compounds should be monitored by the phenol red pH indicator in the mESC medium. In case changes in pH of the mESC medium, pH should be measured and reported.
- During the ToxTracker analysis, changes in cell viability and cell morphology should be monitored after 6h exposure and after 24h, just before preparation of the cells for flow cytometry analysis.

Criteria for a positive ToxTracker result

The ToxTracker assay is considered to have a positive response when a compound induces at least a 2 fold increase in GFP expression in any of the reporters. Activation of the Bscl2-GFP or Rtkn-GFP reporters indicate induction of DNA damage. Btg2 induction is associated with activation of the p53 tumor suppressor. Srxn1-GFP and Blvrb-GFP indicated induction of cellular oxidative stress and Ddit3-GFP activation is associated with the unfolded protein response. Only GFP inductions at compound concentrations that showed <75% cytotoxicity are used for the ToxTracker analysis. Data from measurements >75% cytotoxicity can not be interpreted in a meaningful way and are therefore discarded.

Requirements for repeat experiments

ToxTracker is standard performed in three independent repeats. When the three repeats give the same ToxTracker results, positive or negative, for the various reporters, the analysis can be considered highly reliable and reproducible. In case one of the repeat experiments gives a different results than the other two repeats (two clear positives and one clear negative, or two clear negatives and one clear positive), **a fourth repeat experiment is demanded**. In case the repeat experiments give comparable results that are around the cut-off values for a positive results (2-fold increase in fluorescence), a fourth repeat experiment might be waived.

ANNEX I: Composition of mES cell culture medium

- 450 ml Knockout ES medium (KO-DMEM, Gibco 10829018)
- 50 ml foetal bovine serum (QC-ed and certified for ES cells by Toxys)
- 5 ml Glutamax (Gibco 35050061)
- 5 ml 100 mM Sodium Pyruvate (Gibco 11360039)
- 5 ml Non-essential amino acids (Gibco 11140035)
- 1 ml 50 mM 2-mercaptoethanol (Gibco 31350010)
- 5 ml Penicillin-Streptomycin (10,000 U/mL) (Gibco 15140122)
- 500 µl Leukemia Inhibitory Factor (LIF, Toxys home-made)

ANNEX II: Checklists for the ToxTracker assay

ToxTracker assay data check list

Dose range finding			Checked	Remarks
	- Performed in wild type mES cells - Test in 96-wells plate - Test 11 compound concentrations in 4-fold dilution steps and a vehicle control - Dose range finding performed in absence and presence of S9 - Cytotoxicity calculated as relative cell count after 24 h. exposure			
	Before exposure	Were the cells growing propertly	☐	Do not continue if cells are dying, differentiating or show slow growth rate
		Were the compounds properly dissolved	☐	Only use soluble compound concentrations
		Start dose finding in absence and presence of S9	☐	
	At moment of exposure	Dd you see precipitation of the compound	☐	In case of precipitation, adjust your test concentrations
		Did you perform a cell count at the moment of exposure	☐	Cell count can be used to calculate cytotoxicity based on RPD
	After exposure	Did you notice issues with the untreated control cells	☐	
		Check for precipitation of the compound	☐	Use microscope to assess compound precipitation in the wells. In case of no/limited cytotoxicity, select the maximum compound concentration that does not precipitate as top doses in ToxTracker
		Did you encounter any problems with cell count	☐	
	Data analysis	Calculate cell viability after treatment based on relative cell count	☐	
		Determine the maximum concentration for ToxTracker	☐	Repeat the dose finding in case of "unusual" dose response curve, i.e. survival curve going up and down
		Maximum concentration results in 30-50% viability (50-70% cytotoxicity)	☐	
		Check if compounds are autofluorescent	☐	If yes, than include wt mES cells in the ToxTracker assay to compensate for autofluorescence
	If you did not encounter any issues during the dose range finding and you we able to determine the proper concentration to apply in the ToxTracker assay, please proceed.			

ToxTracker assay data check

ToxTracker assay			Checked	Remarks
	- Performed in 6 ToxTracker reporter cell lines - Include wild type mES cells in the assay in case of autofluorescence of compounds at concentrations that are applied in ToxTracker (for -S9 AND +S9 treatments - Test in 96-wells plates - Test 5 compound concentrations in 2-fold dilution steps and a vehicle control - Toxtracker assay is performed in absence and presence of S9. - Compound concentrations in ToxTracker -S9 can vary from test +S9 - Determine induction of GFP reporter expression using flow cytometry - Cytotoxicity calculated as relative cell count after 24 h. exposure			
	Before exposure	Were the cells growing propertly	☐	Do not continue if cells are dying, differentiating or a show slow growth rate
		Were the compounds properly dissolved (in case you made fresh solutions)	☐	Try to get the compound into solution by warming the solution or sonication in case you see precipitation of the compound after freeze-thawing the solution. If compound is partly soluble, make a homogenous suspension before preparing the dilutions.
		Start dose finding in absence and presence of S9	☐	
	At moment of exposure	Did you see precipitation of the compound	☐	
		Did you perform a cell count at the moment of exposure	☐	Cell count can be used to calculate cytotoxicity based on RPD
		Did you refresh the medium after 3 h. for the +S9 exposures	☐	
	After exposure	Did you notice issues with the untreated control cells	☐	
		Did you encounter any problems with analysis of the ToxTracker reporters by flow cytometry	☐	
	Data analysis	Calculate cell viability after treatment based on relative cell count	☐	CYTOTOXICITY IN TOXTRACKER SHOULD BE COMPARABLE TO THE DOSE FINDING. IF NOT, RESULTS SHOULD BE DISCARDED
		Determine the relative induction levels of the different ToxTracker reporters	☐	
		In case of autofluorescence, correct the GFP reporter levels using the fluorescence measurement in wt mES cells	☐	See ToxTracker protocol for instructions how to perform the autofluorescence correction
		Did the assay control compounds provide comparable results as your historical controls	☐	
		Did you get comparable results as from previous repeat experiments	☐	

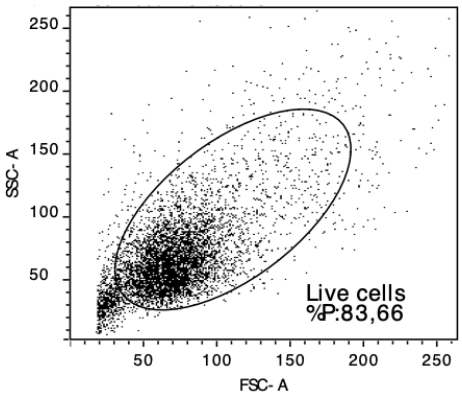
ToxTracker data interpretation

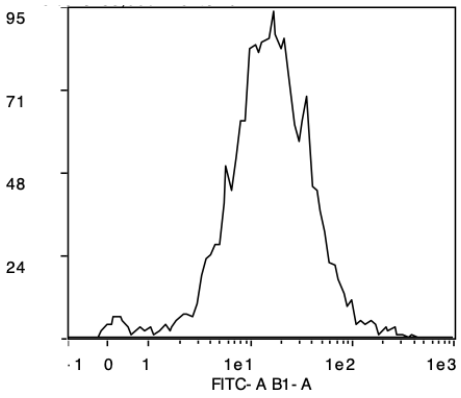
ToxTracker	
Bscl+Rtkn positive	- Compound is classified as genotoxic
	- Activation of Bscl2-GFP indicates DNA reactivity of a compound, induction of bulky DNA lesions and DNA replication stress. Compound likely mutagenic
	- Activation of Rtkn-GFP indicates induction of DNA double strand breaks. Compound likely clastogenic
Rtkn positive, Bscl2 negative	- Compound is classified as genotoxic
	- Compound has potential aneugenic properties
	- Potential indirect genotoxicity related to high levels of oxidative stress
Bscl2+Rtkn negative	- Compound is classified as non-genotoxic
Btg2 positive	- Compound activates the p53 response
	- Compound is potentially genotoxic but only when Bscl2-GFP and/or Rtkn-GFP reporters are activated
Srxn1 and/or Blvrb positive	- Compound induces oxidative stress
Ddit3 positive	- Compound induces the unfolded protein response
	- Induction levels of <2-fold for the GFP reporters should be considered as a negative ToxTracker result
	- Induction of the ToxTracker reporters of >1.5-fold but lower than 2-fold indicates potential (geno)toxic properties but further testing will be required.
	- A >2-fold GFP induction at one concentration is sufficient for a positive ToxTracker result
	- In case no logical dose response is observed, data should be critically reviewed
	- Activation of the Bscl2-GFP and Rtkn-GFP reporters indicates genotoxicity of a compound
	- Activation of the Srxn1-GFP and Blvrb-GFP reporters indicates induction of oxidative stress
	- Activation of the Ddit3 reporter indicates induction of protein damage
	- Activation of the Btg2-GFP reporters indicates induction of the p53 response
	- In case autofluorescence levels of compounds is >10-fold, even after fluorescence compensation ToxTracker results should be interpreted with extreme caution

ANNEX III: Guidance on flow cytometer settings

Setting gates for living cells.

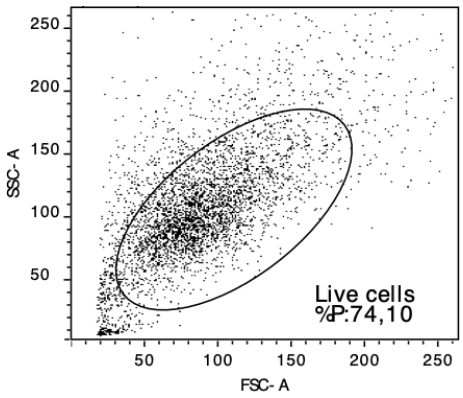
Untreated

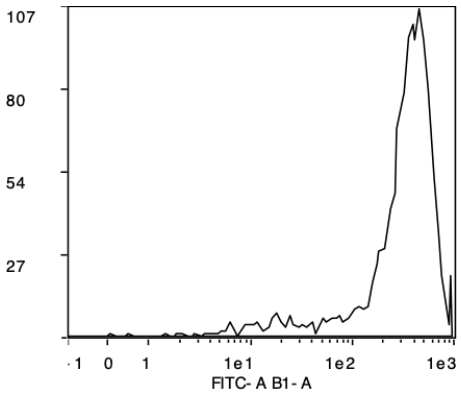




Cisplatin

Make that the intact cells are properly separated from the dead cells and debris. The fluorescence histograms should only be based in the “living cells” gate.





Background GFP levels for each cell line

The proper flow cut-meter settings should be set in a test run, before you start the validation experiments. If you do not get all fluorescence values within the ranges given, please reduce or increase the laser power. Setting will vary between brands and individual flow cytometers.

In the case you obtain much lower levels in untreated cells in 96-well plate please increase the power of the blue laser (GFP signal) until you are around these levels. In case when you are much higher in background levels, please reduce the power of the laser to match the values.

Using laser power settings that result in background too high or too low fluorescence levels in the reporter cell lines can affect the fold changes in ToxTracker reporter activation upon exposure to the test compounds.

	BD Facscanto	Guava	MacsQuant
Bscl2-GFP	300-500	50-80	10-13
Srxn1-GFP	200-400	15-35	4-7
Btg2-GFP	200-400	15-50	4-7
Rtkn-GFP	400-800	90-140	17-24
Blvrb-GFP	400-800	60-110	12-20
Ddit3-GFP	100-300	20-40	4-5

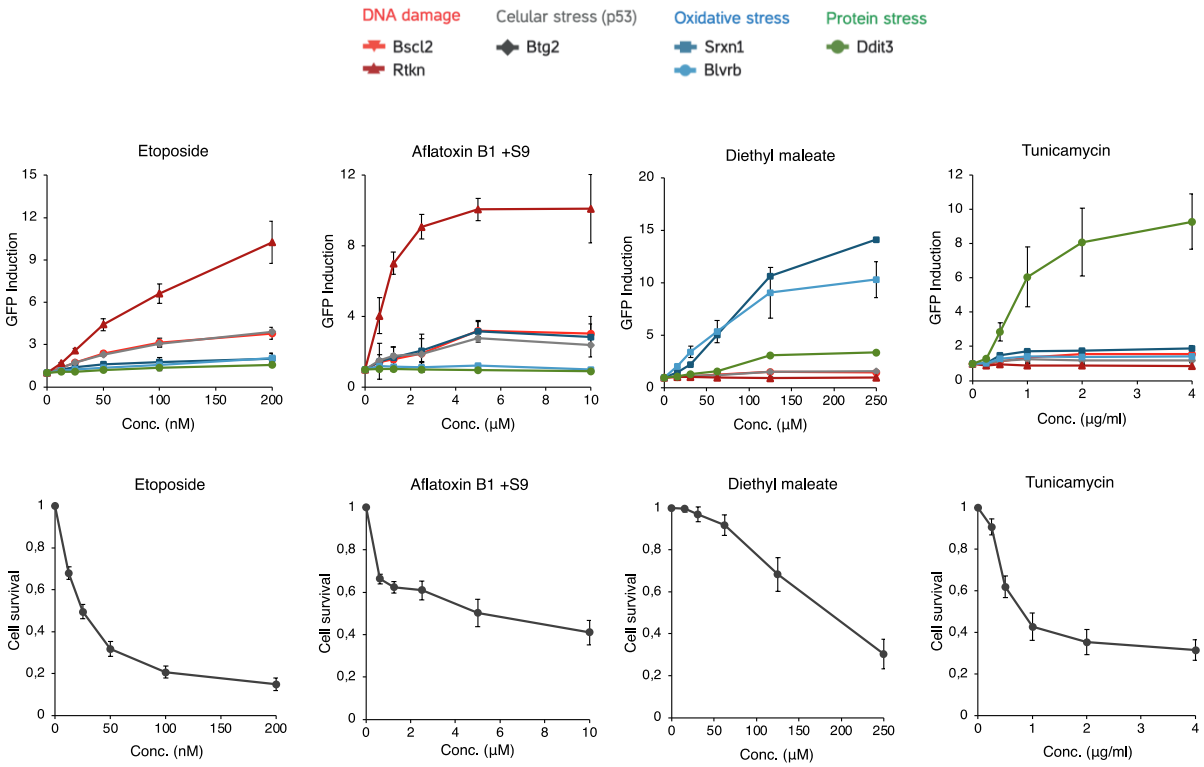
Annex 2: Compound selection for the ToxTracker validation study

	Compound	CAS number	In vitro				In vivo		
			Ames	MLA	MN	CA	MN	CA	TgR
Group I: Genotoxic carcinogens									
1	Etoposide	33419-42-0	P	P	P	P	P	P	N
2	Mitomycin C	50-07-7	P	P	P	P	P	P	P
3	Cisplatin	15663-27-1	P	P	P	P	P	P	P
4	1,2- Dimethylhydrazine	306-37-6	P				P		
5	1,2-dibromoethane	106-93-4	P	P		P	E	N	N
6	Cyclophosphamide	6055-19-2	P	P	P	P	P		P
7	2-Acetylaminofluorene	53-96-3	P	P	P	P	P	P	P
8	Azidothymidine (Zidovudine)	30516-87-1	P	P	P	P	P		
9	ENU	759-73-9	P	P	P	P	P	P	P
10	Acrylonitrile	107-13-1	P	P		P	E	N	N
11	Benzene	71-43-2	N		P	P	P	P	P
12	4,4′ -Oxydianiline	101-80-4	P	P		P	P	P	
13	Busulfan (Myleran)	55-98-1	P	P	P	P	P	P	
14	Ethyl methanesulfonate	62-50-0	P	P	P	P	P	P	P
15	p-Chloroaniline	106-47-8	P	E		P	P		N
16	7,12-Dimethyl-benzanthracene	57-97-6	P	P	P	P	P	P	P
17	Benzo[a]pyrene	50-32-8	P	P	P	P	P	P	P
18	Cadmium Chloride	10108-64-2	E		P	P	P	P	
19	Dimethylnitrosamine	62-75-9	P	P	P	P	P		P
20	2,4-Diaminotoluene	95-80-7	P	P		P	N		P
21	o-Anisidine	90-04-0	P	P		P	N		P
22	4-nitroquinoline-1-oxide	56-57-5	P	P	P	P	P	P	P
Group II: Genotoxic non-carcinogens									
23	6-Mercaptopurine	50-44-2	P	P	P	P	P		
24	Cytosine arabinose	147-94-4	E	P	P	P	P	P	
25	p-Phenylenediamine 2HCl	624-18-0	P	P	P	P	N		
26	8-Hydroxyquinoline	148-24-3	P			P	N		
27	9-Aminoacridine	90-45-9							
28	2,6-Diaminotoluene	823-40-5	P		P	P	P	N	N
29	3-Nitropropionic acid	504-88-1	P	P	N	E			
30	p-Anisidine	104-94-9	P	E		P			
31	5-fluorouracil	51-21-8	N		P	P	P		
32	Phenol	108-95-2	N		P	P	P	N	
Group III: Non-genotoxic carcinogens									

33	Di(2-ethylhexyl)phthalate	117-81-7	N		N	N	N	N	E
34	Lead (ii) acetate trihydrage	6080-56-4	N		P	E	E	P	
35	2-Phenylphenol sodium salt	6152-33-6	P			E	N	N	
36	Ropinirole hydrochloride	91374-20-8	N	N		N	N		
37	Methyl carbamate	598-55-0	N			N	N		
38	Cyclosporin A (CsA)	59865-13-3	N				N		
39	Sodium saccharin	128-44-9	N	N	N	N	N	N	N
40	Diethanolamine	111-42-2	N			N	N		
41	Hexachloroethane	67-72-1	N		N	N	N		
42	Melamine	108-78-1	N			N	N		
Group IV: Non-genotoxic non-carcinogens									
43	Tunicamycin	11089-65-9	N		P		N		
44	p-Nitrophenol (4-nitrophenol)	100-02-7	N		E	P	N		
45	Phenanthrene	85-01-8	P		E	E			
46	Tertiarybutylhydroquinone	1948-33-0	N		P	P	N	N	
47	Benzyl alcohol	100-51-6	N		N	N	N		
48	Vanilin	121-33-5	N			N	N		
49	Erythromycin	114-07-8	N			N			
50	Sodium diclofenac	15307-79-6	N	N		N	N	N	
51	o-anthranilic acid	118-92-3	N		N	N	N	N	
52	Tolbutamide	64-77-7	N			N	P		
53	2-ethyl-1,3-hexanediol	94-96-2	N	N	N	P	N	N	
54	Chlorpheniramine maleate	113-92-8	N	P		P	N		
55	Ampicillin trihydrate	7177-48-2	N			N	N	P	
56	Sodium chloride	7647-14-5							
57	D-mannitol	69-65-8	N			N	N	N	
58	Allyl alcohol	107-18-6	P	P		P	N		
59	(2-chloroethyl)trimethyl-ammonium chloride	999-81-5	N			N		N	
60	Sulfisoxazole	127-69-5	N		N	N	N	N	
61	Sucrose	57-50-1	N				N		
62	Cyclohexanone	108-94-1	N			P		E	
63	1-Nitropropane	108-03-2	N			N	N		
64	Phenformin HCl	834-28-6	N			N			

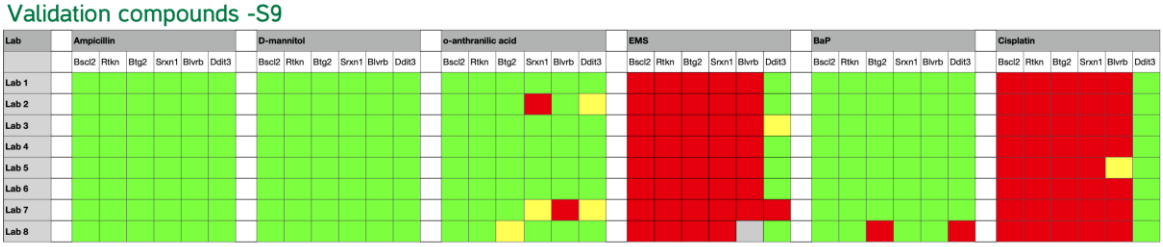
Annex 3: Example ToxTracker data analysis template

Annex 4: Example data set from ToxTracker installation training

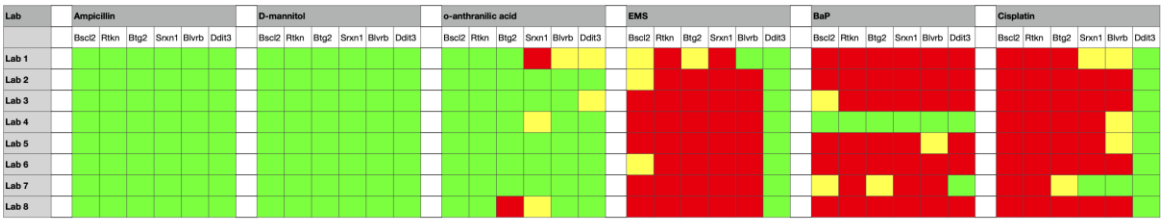


Annex 5: Results from phase 2 proficiency testing of the ToxTracker validation

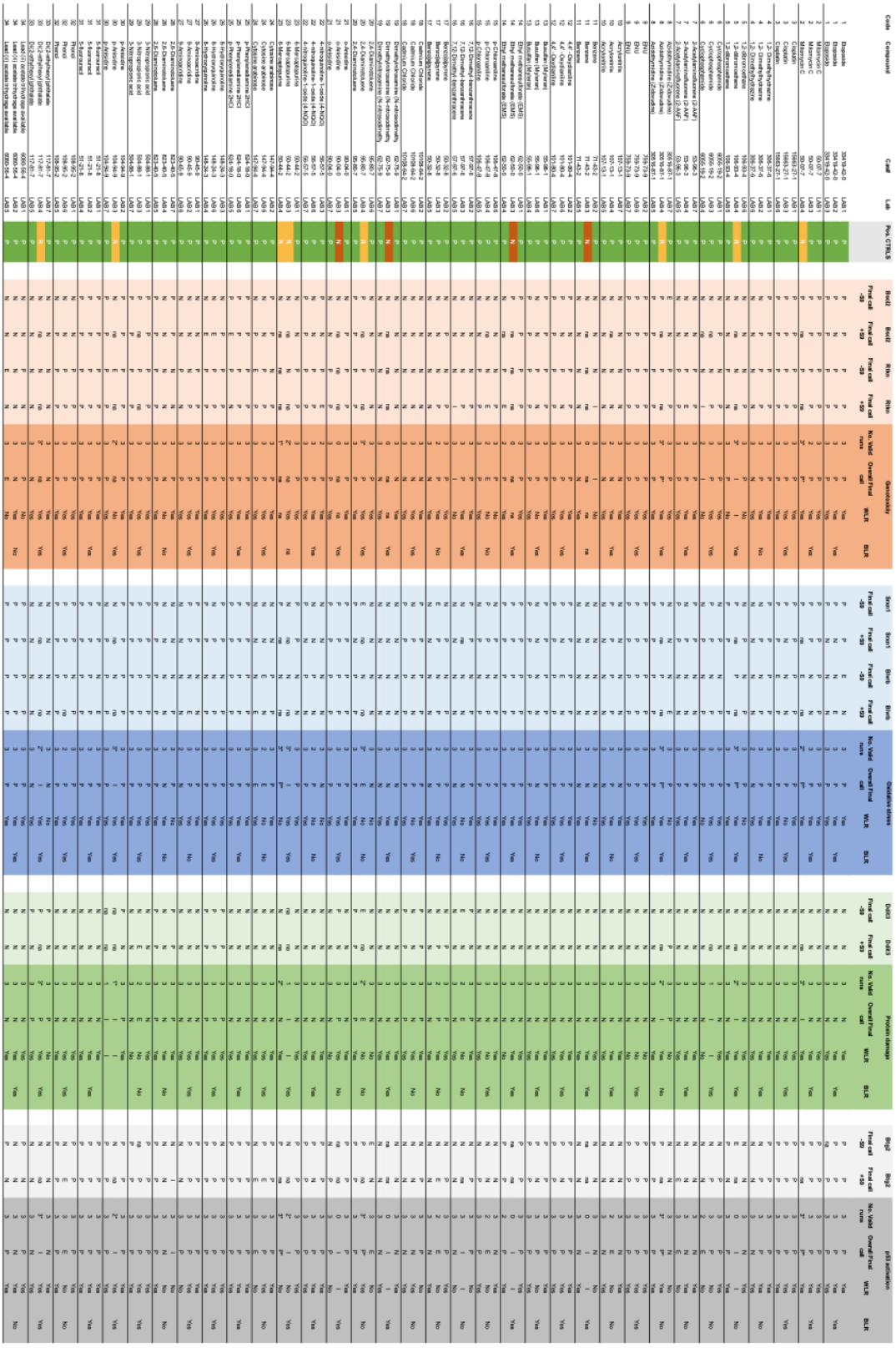
Validation compounds -S9



Validation compounds +S9



Annex 6: Summary of the ToxTracker validation results for all reporters



CONTROLS	
P	Acceptable: proof that reporter cell lines provide responses within the historical control range, proof that the S9-metabolizing system works sufficiently
R	Acceptable with restrictions: proof that reporter cell lines provide responses within the historical control range, but only acceptable data without S9
N	No acceptable data: sufficient evidence that the reporter cell lines show responses within the historical control range
*	Only data for test +S9