Interlaboratory validation of the ToxTracker genotoxicity reporter assay.

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Introduction

ToxTracker is a mammalian stem cell-based reporter assay that detects various hazardous chemical properties that have been associated with increased cancer risk. ToxTracker contains six different GFP-tagged reporters that monitor the activation of specific cellular signaling pathways upon exposure to compounds, thereby allowing the discrimination between induction of DNA damage, oxidative stress and protein damage (Hendriks et al, Tox Sci 2016).

Results

To investigate the reliability and transferability of the ToxTracker assay, we performed a limited interlaboratory validation study. In total 27 compounds (24 test compounds and 3 assay controls) were tested at least three times by two independent laboratories. The within-laboratory reproducibility (WLR) for the six different ToxTracker reporters was assessed per category of reporters (DNA damage, oxidative stress, protein damage) and for all reporters together. The overall WLR was on average 94% for both laboratories. Also the between-laboratory reproducibility (BLR) was evaluated for the different reporters with the highest concordance for the genotoxicity reporters (96.3%), followed by the reporters for p53 activation (92.6%), oxidative stress (88.0%) and protein damage 85.2%. This resulted in an overall BLR of 90.5%. The predictive capacity for genotoxicity was calculated for each laboratory and for the cumulative results of the two laboratories. The sensitivity for identification of genotoxic compounds was 77.8% (7 out of 9 genotoxic compounds tested positive in ToxTracker). The specificity was 94.1% (16 out of 17 non-genotoxic compounds tested negative in ToxTracker for both laboratories). In this study, the overall concordance between ToxTracker and the standard battery of in vitro and in vivo genotoxicity tests was 85%.

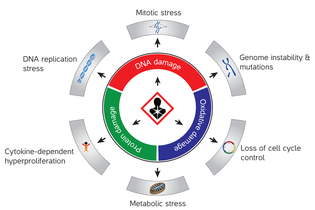
Conclusion

The interlaboratory validation indicates an excellent transferability and predictive capacity of the ToxTracker assay. Furthermore, the combination of the six GFP reporter cell lines can be applied to gain insight into the mechanisms of genotoxicity.

The ToxTracker reporter assay

- Stem cell-based reporter assay
- *In vitro* carcinogenicty hazard screening
- Insight into mechanisms of genotoxicity

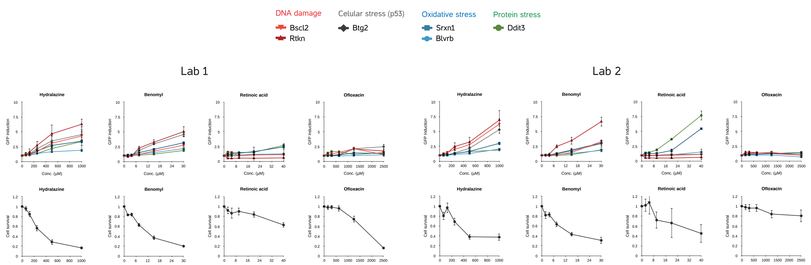
- Six independent GFP reporter cell lines
- High throughput detection by flow cytometry
- Discriminate between induction of DNA damage, oxidative stress and protein damage



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

Test name	Sensitivity (%)	Specificity (%)
Regulatory		
Bacterial reversion (Ames)	60	77
Chromosome aberrations	70	55
Mammalian mutation	81	48
Screening		
ToxTracker	94	95
Ames MPF	58	63
GreenScreen HC	87	95

Validation of the ToxTracker assay



Selective activation of the ToxTracker reporter cell lines. GFP reporter cells were exposed to increasing concentrations of four test compounds. The test compounds were tested in two independent laboratories. GFP induction levels in intact cells were determined by flow cytometry at 24 h. after initiation of the exposure. The relative cell survival was determined by cell count after 24 h. exposure using flow cytometry. Graphs show the summary of three independent repeat experiments.

Between-laboratory reproducibility

	Genotoxicity (Bscl2/Rtkn)			Oxidative stress (Srxn1/Blvrb)			Protein Damage (Ddit3)			p53 activation (Btg2)		
	Lab 1	Lab 2	Concordance	Lab 1	Lab 2	Concordance	Lab 1	Lab 2	Concordance	Lab 1	Lab 2	Concordance
Cisplatin	pos	pos	yes	pos	pos	yes	neg	neg	yes	pos	pos	yes
5-Fluorouracil	pos	pos	yes	neg	neg	no	neg	neg	yes	pos	neg	no
Hydralazine	pos	pos	yes	neg	pos	no	neg	neg	yes	pos	pos	yes
Benomyl	pos	pos	yes	pos	pos	yes	neg	neg	yes	pos	pos	yes
1-Nitropyrene	pos	pos	yes	pos	pos	yes	pos	pos	yes	yes	pos	yes
2,7-Dinitrofluorene	neg	neg	yes	neg	neg	yes	neg	neg	yes	neg	neg	yes
Noscapine HCl	pos	pos	yes	eq	pos	eq	neg	neg	yes	pos	pos	yes
Norethynodrel	pos	pos	yes	pos	pos	yes	neg	neg	yes	pos	pos	yes
Nitrofurantoin	neg	neg	yes	pos	pos	yes	pos	pos	yes	neg	neg	yes
Alachlor	neg	neg	yes	pos	pos	yes	neg	pos	no	neg	neg	yes
Chloramphenicol	neg	neg	yes	eq	pos	eq	pos	pos	yes	neg	neg	yes
Atrazine	neg	neg	yes	pos	neg	no	pos	neg	no	neg	neg	yes
Bisphenol A	neg	neg	yes	neg	pos	no	pos	pos	yes	neg	neg	yes
Bromobenzene	neg	neg	yes	neg	neg	yes	neg	neg	yes	neg	neg	yes
Cannrenoate K+	neg	neg	yes	neg	neg	yes	neg	neg	yes	neg	neg	yes
Clomiphene	neg	neg	yes	neg	neg	yes	neg	neg	yes	neg	neg	yes
Dazomet	neg	neg	yes	pos	pos	yes	pos	pos	yes	neg	neg	yes
Diethyl maleate	neg	neg	yes	pos	pos	yes	neg	pos	no	neg	neg	yes
Dexamethasone	neg	neg	yes	neg	neg	yes	pos	neg	no	neg	neg	yes
Endosulfan	neg	neg	yes	pos	pos	yes	pos	pos	yes	neg	neg	yes
Nicotine	neg	neg	yes	neg	neg	yes	neg	neg	yes	neg	neg	yes
Ofloxacin	pos	pos	no	neg	neg	yes	neg	neg	yes	pos	neg	no
Retinoic acid	neg	neg	yes	pos	pos	yes	pos	pos	yes	neg	neg	yes
Salicylamide	neg	neg	yes	neg	neg	yes	neg	neg	yes	neg	neg	yes
Testosterone	pos	pos	yes	pos	pos	yes	neg	neg	yes	pos	pos	yes
Tunicamicin	pos	pos	yes	neg	neg	yes	pos	pos	yes	neg	neg	yes
Urea	neg	neg	yes	neg	neg	yes	neg	neg	yes	neg	neg	yes
Total Concordance			**96%**			**84%**			**85%**			**93%**

The between-laboratory reproducibility for the two validation laboratories. The BLR was assessed per category of reporters. For the evaluation of the BLR the rule of majority voting was applied, e.g. a chemical that resulted in a positive response in 2 out of 3 runs for a GFP reporter was predicted positive for that reporter. Cells with gray background indicate inconclusive results, cells with an orange background indicate an overall positive prediction, and cells with a green background indicate an overall negative prediction. The overall BRL for the two laboratories was 90.5%.

Outline of the validation study

- Validation in two independent laboratories
- Determine within-laboratory (WLR) and between-laboratory reproducibility (BLR)
- Determine sensitivity and specificity of ToxTracker for genotoxicity testing
- 24 compounds + 3 assay control compounds

Genotoxic	Non-genotoxic	
5-Fluorouracil	Alachlor	Dexamethasone
Hydralazine	Chloramphenicol	Endosulfan
Benomyl	Atrazine	Nicotine
1-Nitropyrene	Bisphenol A	Ofloxacin
2,7-Dinitrofluorene	Bromobenzene	Retinoic acid
Noscapine HCl	Cannrenoate K+	Salicylamide
Norethynodrel	Clomiphene	Testosterone
Nitrofurantoin	Dazomet	Urea

Assay controls:

Cisplatin	Diethyl maleate	Tunicamicin

Validation protocol

- Each lab tests 24 compound
- Test compounds at 5 conc. in 2-fold dilution steps
- Expose ToxTracker cell lines for 24 h.
- Detect induction of GFP reporters by flow cytometry
- Determine cytotoxicity by relative cell count
- Three independent repeat experiments
- Independent statistical analysis
- Calculate WLR and BLR
- Calculate sensitivity and specificity

Within-laboratory reproducibility

Lab 1

	Genotoxicity				Oxidative Stress				Protein damage				p53 Activation			
	Bscl2		Rtkn		Srxn1		Blvrb		Ddit3				Btg2			
				WLR				WLR				WLR				WLR
Cisplatin	68	70	1313	100%	57	57	1454	100%	57	57	100%	57	57	100%	57	100%
5-Fluorouracil	33	33	66	100%	33	33	66	100%	33	33	100%	33	33	100%	33	100%
Hydralazine	44	44	88	100%	104	104	858	100%	104	104	75%	44	104	100%	44	100%
Benomyl	33	33	38	83.30%	33	33	466	67%	33	33	100%	33	33	100%	33	100%
1-Nitropyrene	33	33	66	100%	33	33	66	100%	33	33	100%	33	33	100%	33	100%
2,7-Dinitrofluorene	33	33	66	100%	33	33	66	100%	33	33	100%	33	33	100%	33	100%
Noscapine HCl	104	104	8	75%	104	104	8	75%	33	33	100%	104	104	100%	104	100%
Norethynodrel	104	104	78	87.50%	104	104	858	100%	104	104	100%	104	104	100%	104	100%
Nitrofurantoin	104	104	858	100%	104	104	858	88%	33	33	67%	104	104	100%	104	100%
Alachlor	33	33	66	100%	33	33	66	100%	33	33	100%	33	33	100%	33	100%
Chloramphenicol	104	104	858	100%	104	104	858	75%	104	104	100%	104	104	100%	104	100%
Atrazine	33	33	66	100%	33	33	66	100%	33	33	100%	33	33	100%	33	100%
Bisphenol A	33	33	66	100%	33	33	66	83%	33	33	100%	33	33	100%	33	100%
Bromobenzene	33	33	66	100%	33	33	66	100%	33	33	100%	33	33	100%	33	100%
Cannrenoate K+	33	33	66	100%	33	33	66	100%	33	33	100%	33	33	100%	33	100%
Clomiphene	104	104	858	75%	104	104	858	75%	104	104	100%	104	104	100%	104	75%
Dazomet	33	33	66	100%	33	33	66	67%	33	33	67%	33	33	100%	33	100%
Diethyl maleate	57	57	1454	100%	57	57	1454	93%	57	57	86%	57	57	87%	57	57%
Dexamethasone	33	33	66	100%	33	33	66	100%	33	33	100%	33	33	100%	33	100%
Endosulfan	33	33	66	100%	33	33	66	83%	33	33	100%	33	33	100%	33	100%
Nicotine	33	33	66	100%	33	33	66	100%	33	33	100%	33	33	100%	33	67%
Ofloxacin	33	33	66	66.67%	33	33	66	100%	33	33	100%	33	33	100%	33	100%
Retinoic acid	33	33	66	100%	33	33	66	83%	33	33	100%	33	33	100%	33	100%
Salicylamide	33	33	66	100%	33	33	66	100%	33	33	100%	33	33	100%	33	100%
Testosterone	104	104	78	87.50%	104	104	858	100%	104	104	100%	104	104	75%	104	100%
Tunicamicin	57	57	1454	100%	57	57	1454	100%	57	57	100%	57	57	100%	57	100%
Urea	104	104	858	100%	104	104	858	100%	104	104	100%	104	104	100%	104	100%
				95.37%				**92.37%**				**96.08%**				**95.33%**

Lab 2

	Genotoxicity				Oxidative Stress				Protein damage				p53 Activation			
	Bscl2		Rtkn		Srxn1		Blvrb		Ddit3				Btg2			
				WLR				WLR				WLR				WLR
Cisplatin	99	99	1717	100%	99	99	1717	94%	99	99	100%	99	99	100%	99	100%
5-Fluorouracil	33	33	66	100%	33	33	66	100%	33	33	100%	33	33	67%	33	67%
Hydralazine	33	33	66	83%	33	33	66	67%	33	33	67%	33	33	67%	33	100%
Benomyl	33	33	66	83%	33	33	66	100%	33	33	67%	33	33	67%	33	67%
1-Nitropyrene	33	33	66	100%	33	33	66	100%	33	33	100%	33	33	100%	33	100%
2,7-Dinitrofluorene	33	33	66	100%	33	33	66	100%	33	33	100%	33	33	100%	33	100%
Noscapine HCl	33	33	66	100%	33	33	66	100%	33	33	100%	33	33	100%	33	100%
Norethynodrel	33	33	66	83%	33	33	66	100%	33	33	100%	33	33	100%	33	100%
Nitrofurantoin	33	33	66	100%	33	33	66	100%	33	33	100%	33	33	100%	33	100%
Alachlor	33	33	66	100%	33	33	66	100%	33	33	100%	33	33	100%	33	100%
Chloramphenicol	33	33	66	83%	33	33	66	100%	33	33	100%	33	33	100%	33	100%
Atrazine	33	33	66	100%	33	33	66	100%	33	33	100%	33	33	100%	33	100%
Bisphenol A	33	33	66	100%	33	33	66	100%	33	33	100%	33	33	100%	33	100%
Bromobenzene	33	33	66	100%	33	33	66	100%	33	33	100%	33	33	100%	33	100%
Cannrenoate K+	33	33	66	83%	33	33	66	100%	33	33	100%	33	33	100%	33	100%
Clomiphene	33	33	66	83%	33	33	66	83%	33	33	67%	33	33	67%	33	100%
Dazomet	33	33	66	100%	33	33	66	100%	33	33	67%	33	33	100%	33	100%
Diethyl maleate	33	33	66	100%	33	33	66	100%	33	33	100%	33	33	100%	33	100%
Dexamethasone	33	33	66	100%	33	33	66	100%	33	33	100%	33	33	100%	33	100%
Endosulfan	33	33	66	100%	33	33	66	100%	33	33	100%	33	33	100%	33	100%
Nicotine	33	33	66	83%	33	33	66	100%	33	33	67%	33	33	67%	33	67%
Ofloxacin	99	99	1718	94%	99	99	1717	100%	99	99	78%	99	99	78%	99	67%
Retinoic acid	33	33	66	100%	33	33	66	83%	33	33	100%	33	33	100%	33	100%
Salicylamide	33	33	66	100%	33	33	66	67%	33	33	100%	33	33	100%	33	100%
Testosterone	33	33	66	100%	33	33	66	83%	33	33	67%	33	33	100%	33	100%
Tunicamycin	33	33	66	100%	33	33	66	100%	33	33	100%	33	33	100%	33	100%
Urea	33	33	66	100%	33	33	66	100%	33	33	100%	33	33	100%	33	100%
Bromobenzene	33	33	66	100%	33	33	66	100%	33	33	100%	33	33	100%	33	100%
				94.86%				**94.20%**				**90.58%**				**92.65%**

The within-laboratory reproducibility for the two validaiton laboratories. The WLR was assessed per category of reporters. For the calculation of the WLR, the majority voting rule was applied. Cells with gray background indicate inconclusive results, cells with an orange background indicate an overall positive prediction, and cells with a green background indicate an overall negative prediction.The overall WRL for lab 1 was 94.2% and for lab 2 93.7%.

Concordance with in vivo genotoxicity

	Lab 1			Lab 2							
	Classification Toxtracker System	Total exp.	Gntx pos	Classification Toxtracker System	Total exp.	Gntx pos	in vitro GNTX[1]	in vivo GNTX[2]	GNTX overall [3]	Concordance[4]	Carcinogenicity
1-Nitropyrene	pos	3	3	pos	3	3	pos	pos	yes	yes	yes
5-Fluorouracil	pos	3	3	pos	3	3	pos	pos	yes	yes	yes
Benomyl	pos	3	3	pos	3	3	pos	pos	yes	yes	yes
Cisplatin	pos	7	7	pos	9	9	pos	pos	yes	yes	yes
Hydralazine	pos	4	4	pos	3	3	pos	pos	yes	yes	yes
Norethynodrel	pos	4	4	pos	3	3	pos	pos	yes	yes	no
Noscapine HCl	pos	3	3	pos	3	3	pos	pos	yes	yes	no
Testosterone	pos	4	4	pos	3	3	neg	neg	no	no	unknown
2,7-Dinitrofluorene	neg	3	0	neg	3	0	pos	pos	yes	no	yes
Alachlor	neg	3	0	neg	3	0	neg	neg	no	yes	yes
Chloramphenicol	neg	4	0	neg	3	1	neg	neg	no	yes	yes
Atrazine	neg	3	0	neg	3	1	neg	neg	no	yes	no
Bisphenol A	neg	3	0	neg	3	1	neg	neg	no	yes	no
Cannrenoate K+	neg	3	0	neg	3	0	neg	neg	no	yes	no
Clomiphene	neg	4	1	neg	3	0	pos	neg	in vitro pos	yes	no
Dazomet	neg	3	0	neg	3	0	neg	neg	no	yes	no
Dexamethasone	neg	3	0	neg	3	0	neg	neg	no	yes	no
Diethyl maleate	neg	7	0	neg	9	1	neg	neg	no	yes	no
Endosulfan	neg	3	0	neg	3	0	neg	neg	no	yes	no
Nicotine	neg	3	0	neg	3	0	neg	neg	no	yes	no
Nitrofurantoin	neg	4	0	neg	3	1	pos	pos	yes	yes	no
Ofloxacin	pos	3	2	neg	3	0	neg	neg	no	yes	no
Retinoic acid	neg	3	0	neg	3	0	neg	neg	no	yes	no
Salicylamide	neg	3	0	neg	3	0	neg	neg	no	yes	no
Tunicamycin	neg	7	0	neg	9	0	neg	neg	no	yes	no
Urea	neg	3	0	neg	3	0	neg	neg	no	yes	no
Bromobenzene	neg	4	0	neg	3	0	neg	neg	no	yes	no
										Total 85.9%	unknown

[1] Ames test, MN, CA (Weight of evidence approach, majority voting)
[2] MN, CA, Comet (Weight of evidence approach)
[3] Based on vitro and in vivo GNTX data
[4] Concordance overall GNTX prediction and ToxTracker classification

The sensitivity and specificity of the ToxTracker assay in the two validation laboratories. The predictive capacity for genotoxicity and carcinogenicity was calculated for each laboratory and for the cumulative results of the two laboratories. The predictive capacity was evaluated for the Bscl2 and Rtkn reporter since they are activated after exposure to genotoxic agents. A chemical was considered positive if one or both of the reporters were activated (> 2 fold induction of the reporter). The calculations were based on the individual prediction for each chemical in each laboratory. Each chemical was tested in at least three runs. The sensitivity for identification of genotoxic compounds was 77.8% (7 out of 9 genotoxic compounds tested positive in ToxTracker) for both labs. The specificity was 96% (16 out of 17 non-genotoxic compounds tested negative in ToxTracker for both laboratories. The overall concordance between ToxTracker and the standard battery of in vivo genotoxicity tests was 88.9%. The predictive capacity for genotoxic carcinogens in this validation study was 83.3% (5 out of 6 genotoxic carcinogens tested positive in ToxTracker).