toxys®

The NAM Company

ToxTracker Discussion: A Potential New Approach Method for Carcinogenicity Testing

ICCVAM MDF PROPOSAL

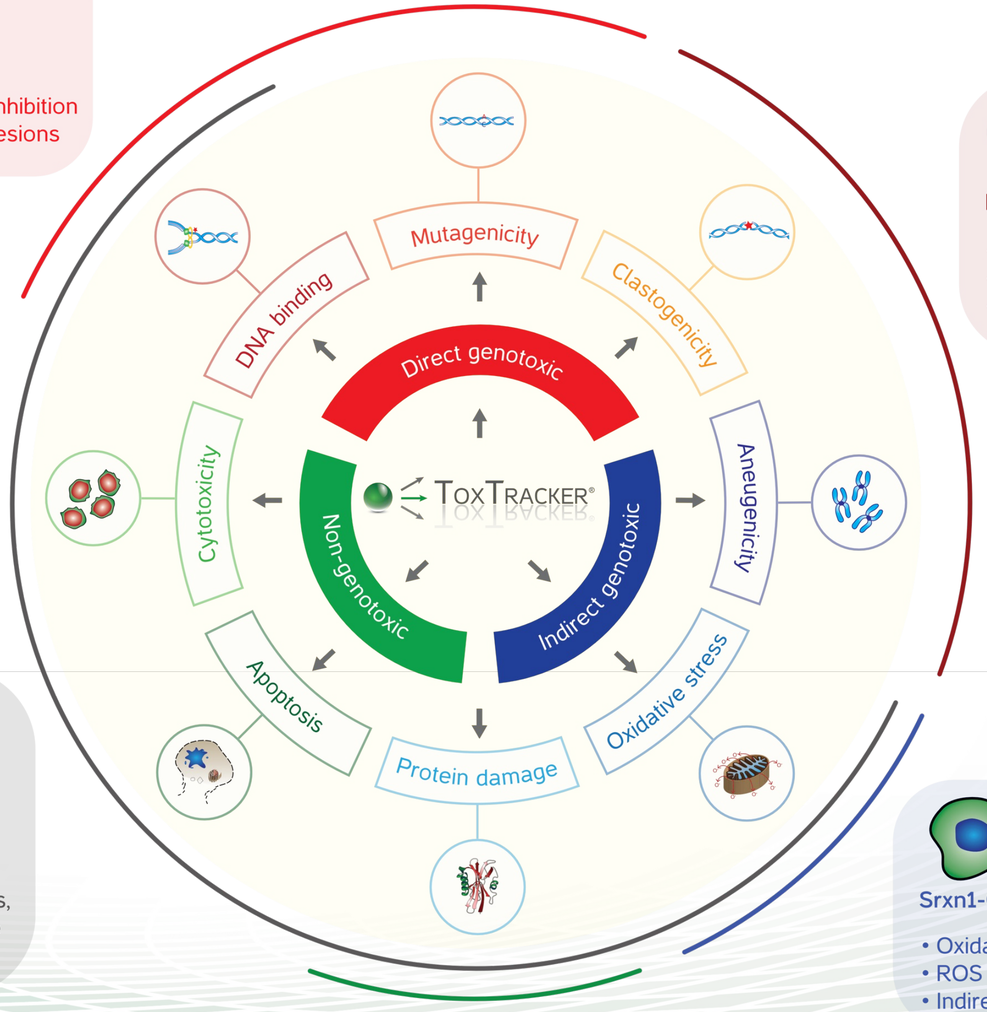
Toxys Inc.
New York, NY

Section 1: ToxTracker Assay

- Born from transcriptomic assessment of genotoxic and non-genotoxic carcinogens.
- Established for identifying direct and indirect genotoxic agents.
- Constructed mouse ES cells with stable BAC GFP-reporters to detect:
  - Bulky Adducts
  - DNA breaks
  - Oxidative stress
  - UPR activation

Bscl2-GFP

- DNA replication inhibition
- Mutagenic DNA lesions



Rtkn-GFP

- DNA double strand breaks
- Aneuploidy (ACE)
- Clastogens vs aneugens

Btg2-GFP

- p53 tumor suppressor activation
- DNA damage, oxidative stress, osmotic shock, ribonucleotide depletion, apoptosis

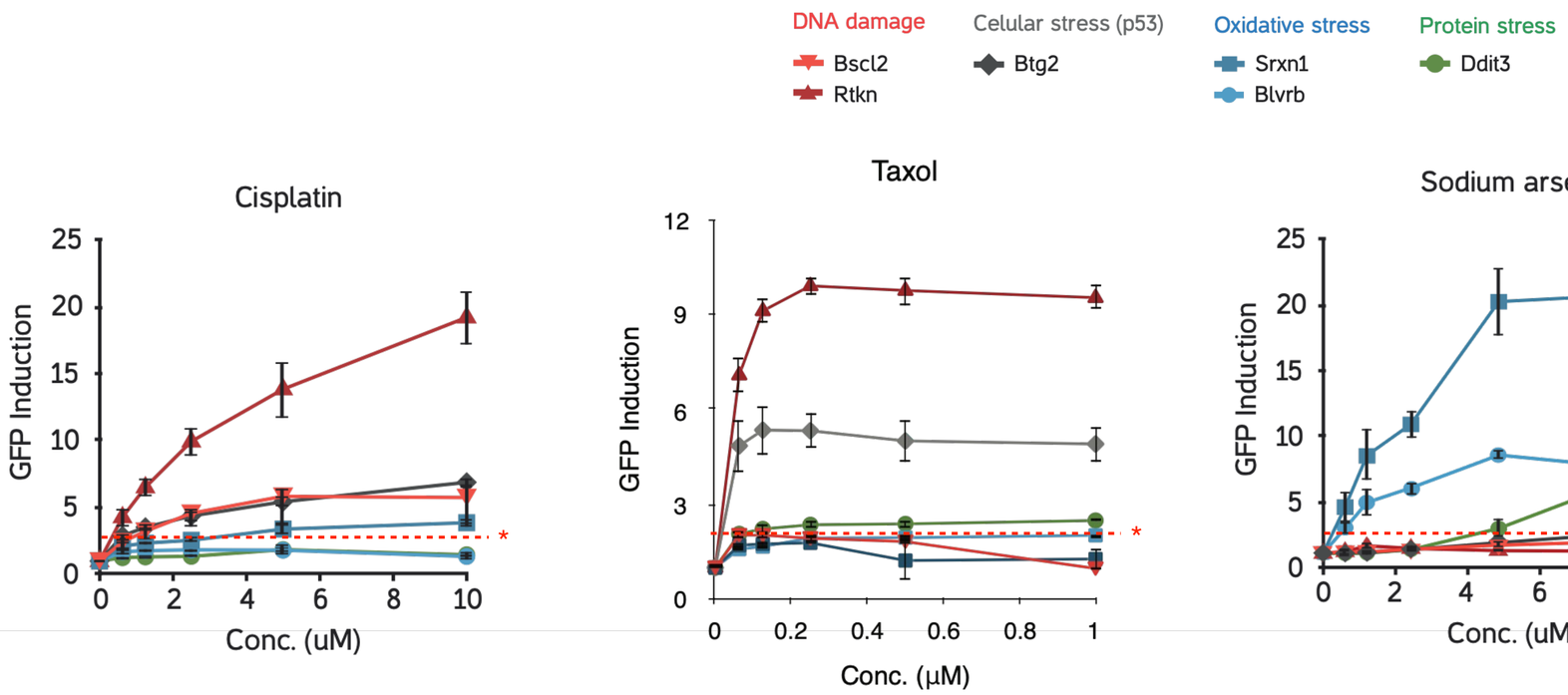
Srxn1-GFP **Blvrb-GFP**

- Oxidative stress
- ROS production
- Indirect genotoxicity (AO)

Ddit3-GFP

- Unfolded protein response
- ER stress

Section 1: Enhanced Understanding



Ames pos.
MN pos.

Ames neg.
MN pos.

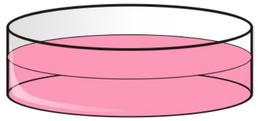
Ames neg.
MN pos.

Ames neg.
MN pos.

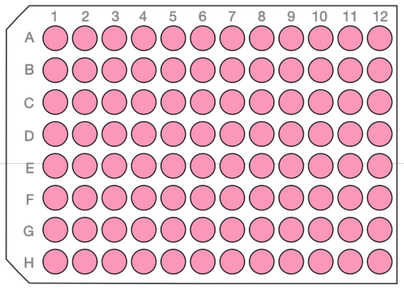
* 2-fold GFP reporter induction is limit for positive ToxTracker result.

Section 1: ToxTracker Dose Range Finding Assay

Day 1

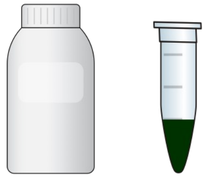


Wild type mouse stem cells



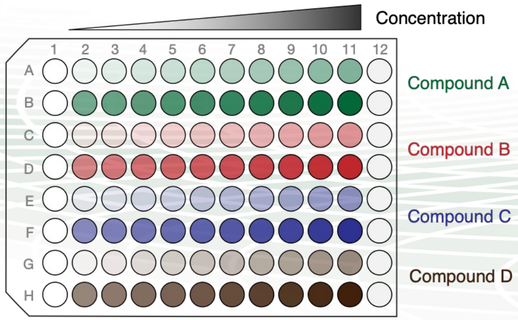
Seed cells in 96-wells plate

Day 2



Dissolve compound in DMSO or H_2O

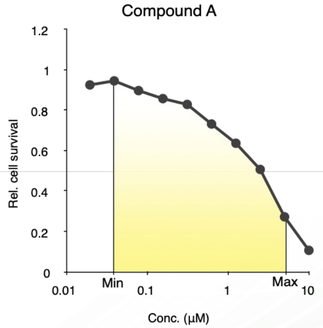
20 serial dilution (in 2-fold)



Expose cells to the compounds (24 h.)

Day 3

Cell count by flow cytometry

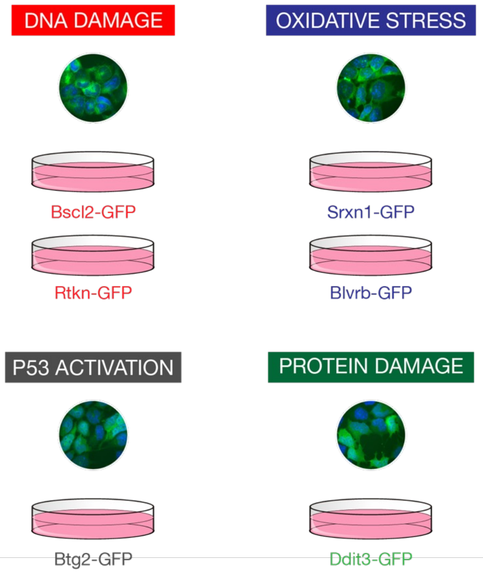


Dose range finding

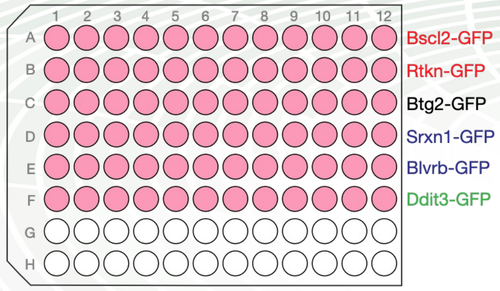
- Cytotoxicity
- Compound solubility
- Autofluorescence
- Metabolic activation (+S9)

Section 1: ToxTracker Definitive Assays

Day 1



Six independent GFP reporter cell lines



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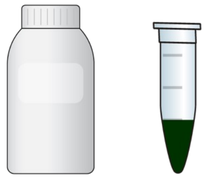

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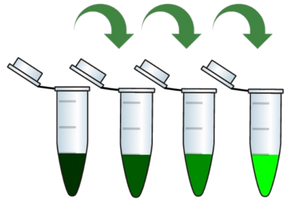
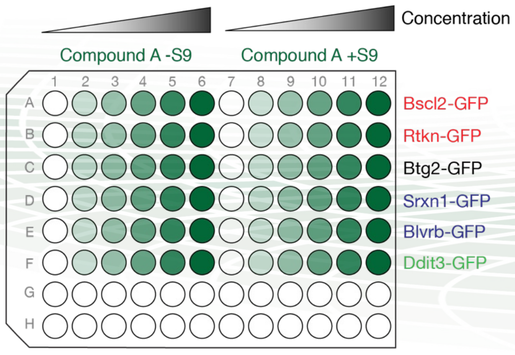


5 serial dilution (in 2-fold)



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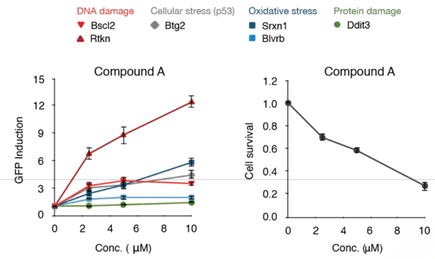
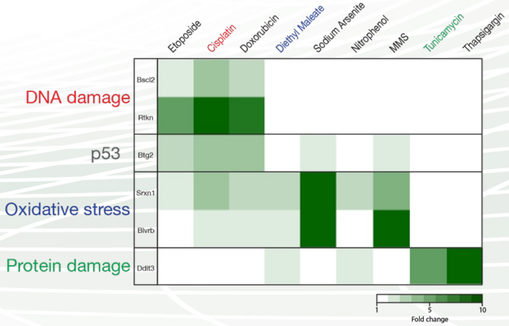
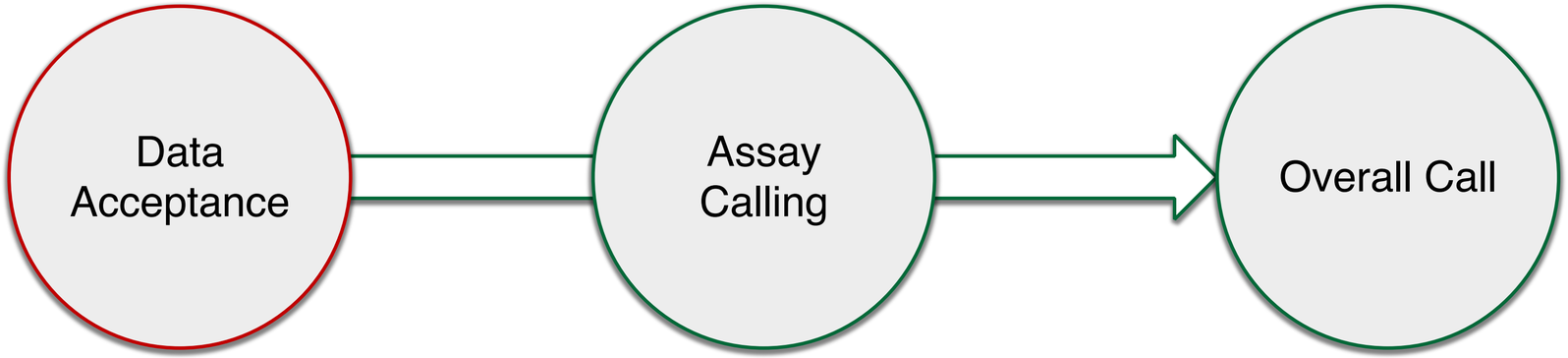




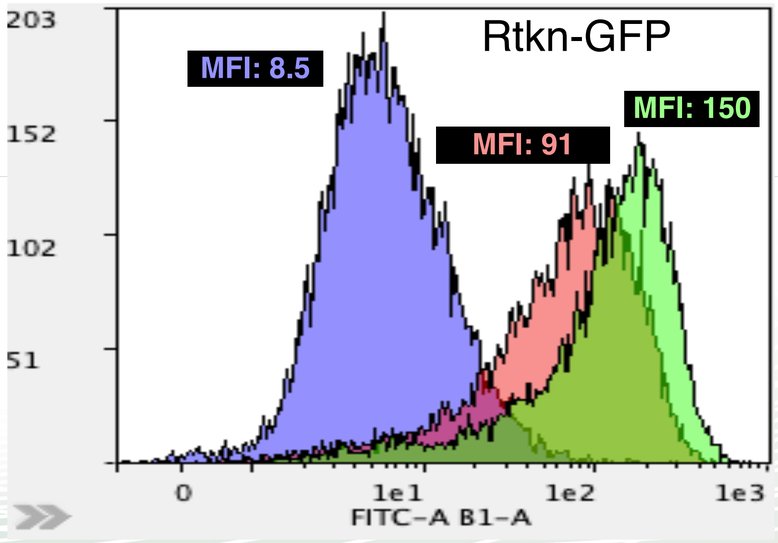
Data analysis using Toxplot software

Section 1: Assay Response Criteria



- **Positive controls (PC) induce meaningful responses**
- Basal (vehicle) GFP fluorescence is within an appropriate control limit
- Autofluorescence is compensated for

PC Response



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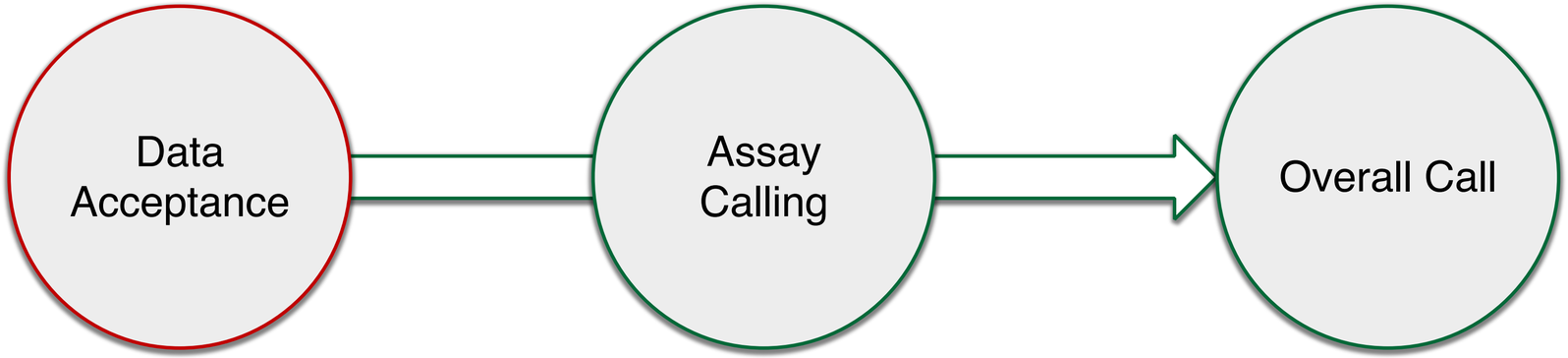

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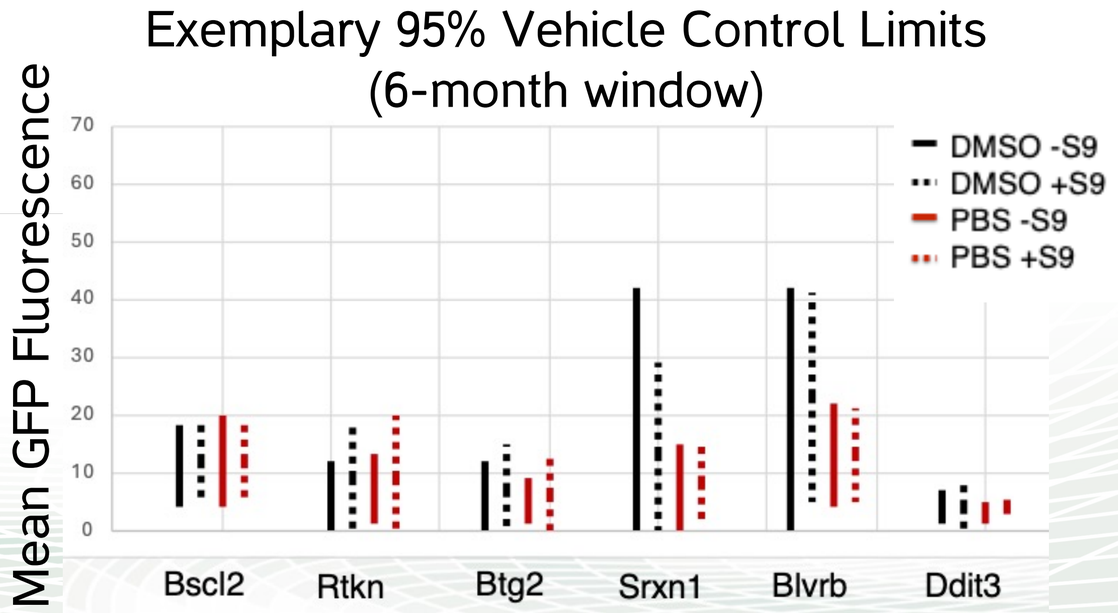

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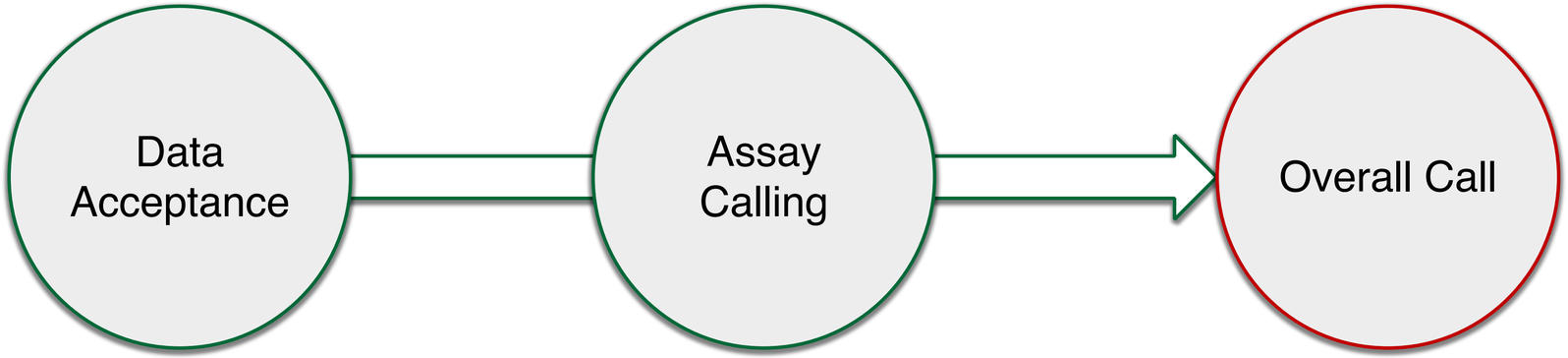
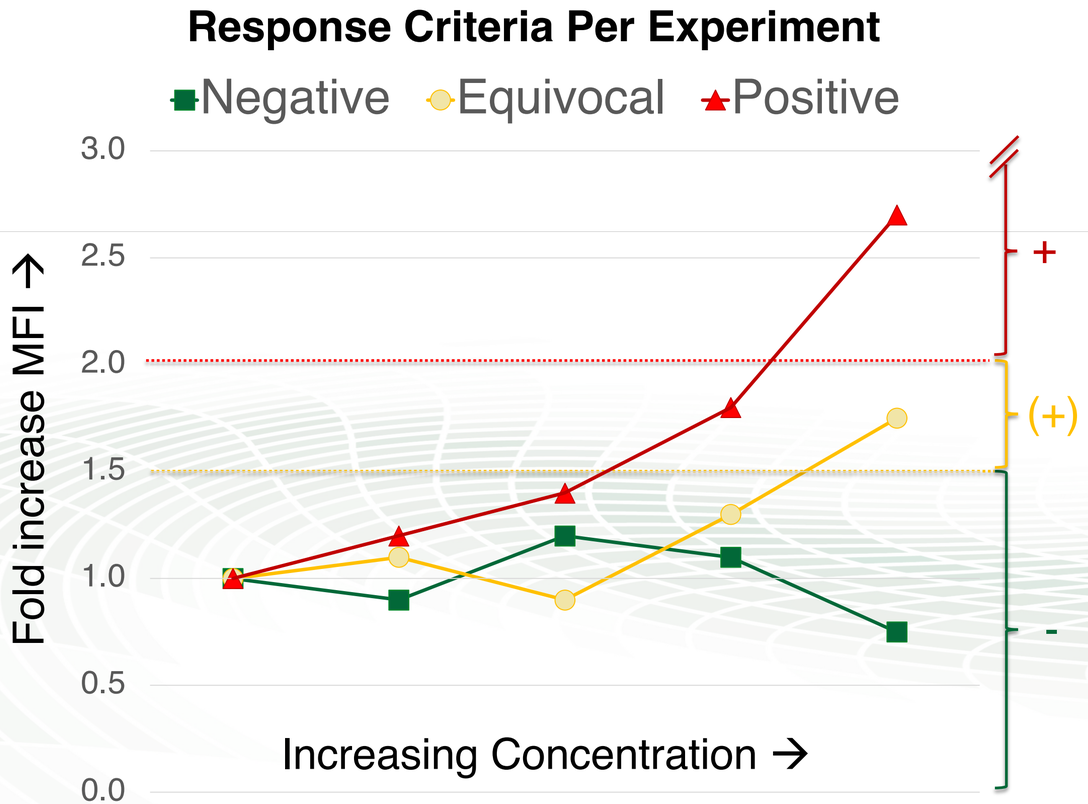


- Consensus calling



Experiment Outcome	Overall Call
+ + +	+
+ + (+)	+
+ + -	+
+ (+) (+)	+
- - -	-
- - (+)	-
- - +	-
- (+) (+)	-
(+) (+) (+)	E
+ (+) -	E

Section 1: Practical Facts

Compound requirement

- Pharmaceuticals (top concentration 1 mM): 5-10 mg
- Chemicals (top concentration 10 mM): 50-100 mg

Turn around time

- Standard service: 2-3 weeks
- Express service: 3 days

Throughput

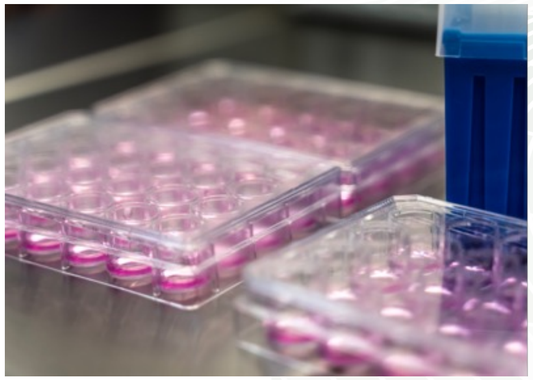
- Standard full test (dose finding, three repeats -/+ S9): 1-25 compounds/week

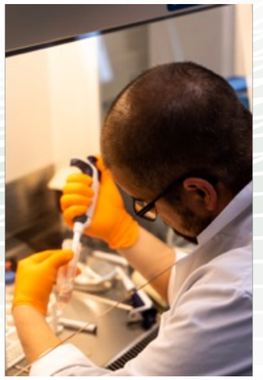
Type of solvents

- DMSO/PBS
- Water
- Ethanol

Types of materials that have been tested

- Small molecules
- Polymers
- Complex mixtures
- Nanomaterial
- Intermediates (occupational toxicology)

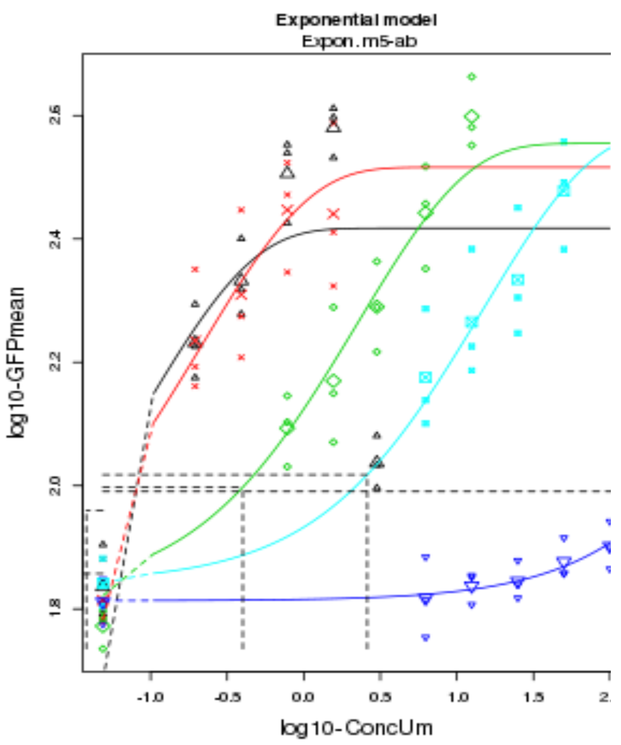




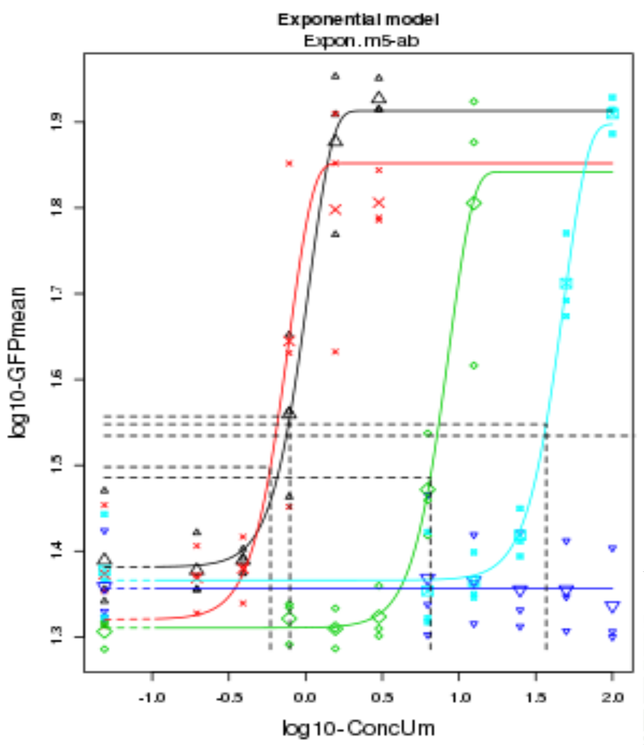

Section 2: Context of use

- ToxTracker is currently used to identify genotoxic hazards and determine mode-of-action (MOA).
- Used to discriminate direct genotoxicants from those with an indirect mechanism of action (e.g., oxidative stress inducers).
- Since direct (DNA-reactive) and indirect genotoxic effects may initiate the carcinogenic process, quantitative methods have been used to assess potential safety margins

Oxidative stress (Blvrb-GFP)



Protein damage (Ddit3-GFP)

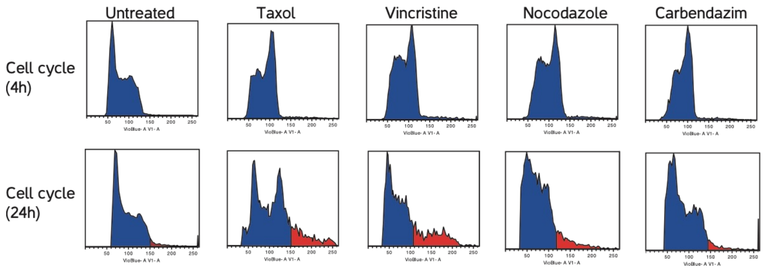


Section 2: MOA with ToxTracker ACE

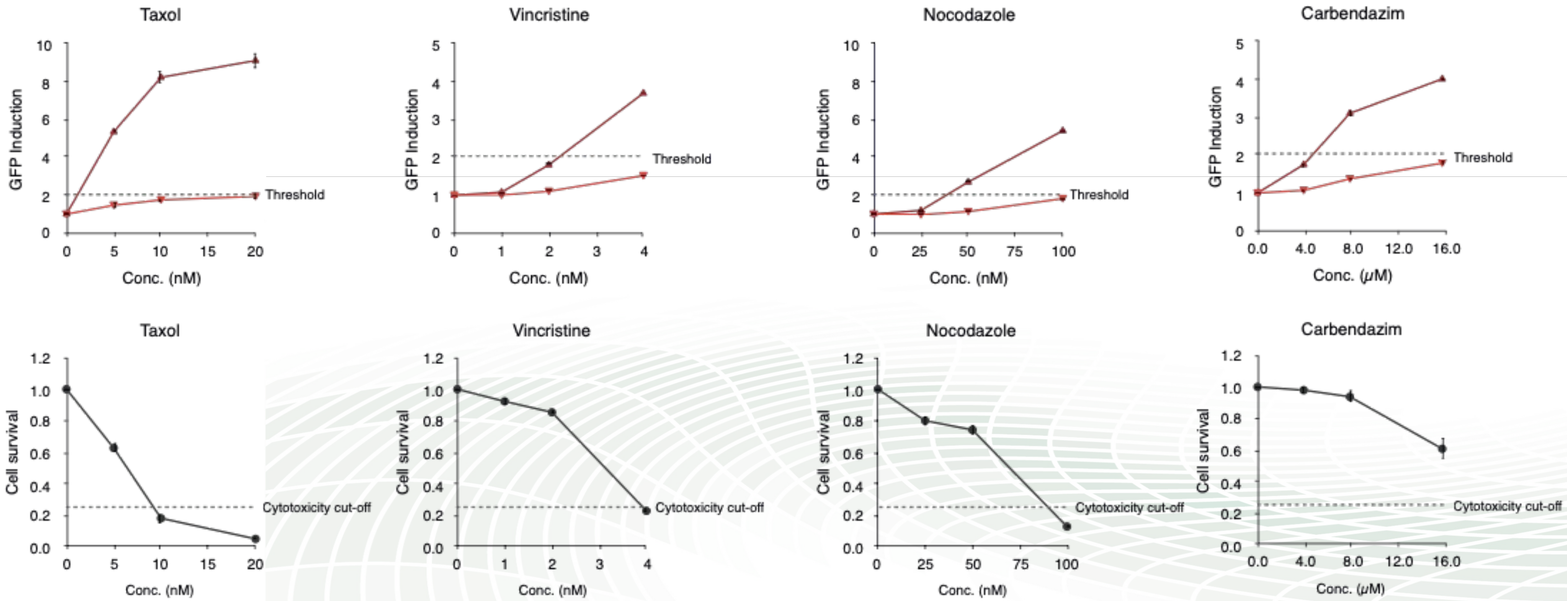
- Multiplexed DNA staining after 4h and 24h exposure
- Determine cell cycle distribution and polyploid induction
- Aneugens have a 4 Hr G2/M block and 24 Hr >5% polyploidy

Aneugens – Tubulin poisons

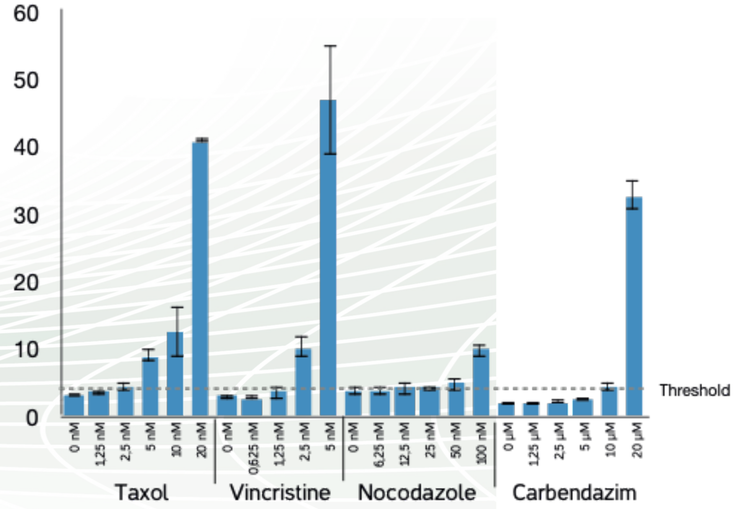
Cell cycle



ToxTracker

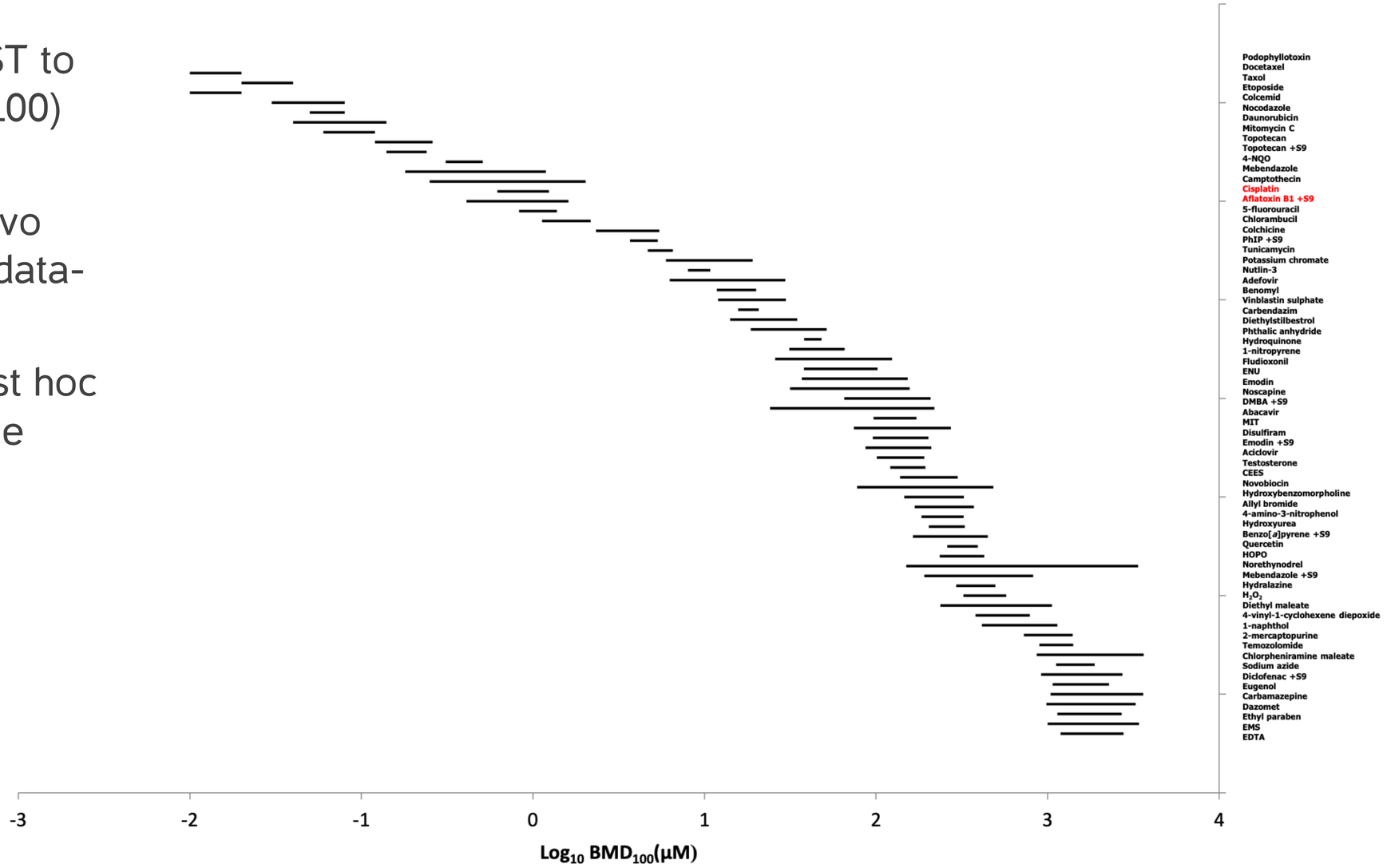


Aneuploidy



Section 2: Quantitative analysis of ToxTracker data

- Analysis using PROAST to calculate BMDs (BMR100)
- Potency ranking for prioritizing further in vivo testing, especially for data-poor compounds
- Has been included post hoc to assess the reference chemicals used during Genetox21 (EPA).



Section 2: Context of Use Summary

Hazard ID

- 384-well, Rtkn and Bscl2 cell lines – quick genotoxic predictions
- Full panel of cell lines to classify MOA

Risk Assessment

- Amenable to BMD analysis, and deriving AEDs
- Quantitative PODs overlap with those derived from in vivo studies

3R's Aligned

- As a stand-alone assay, better predicts genotoxic carcinogens than other in vitro tools, providing trust for moving away from animal testing

Modalities/Products Tested

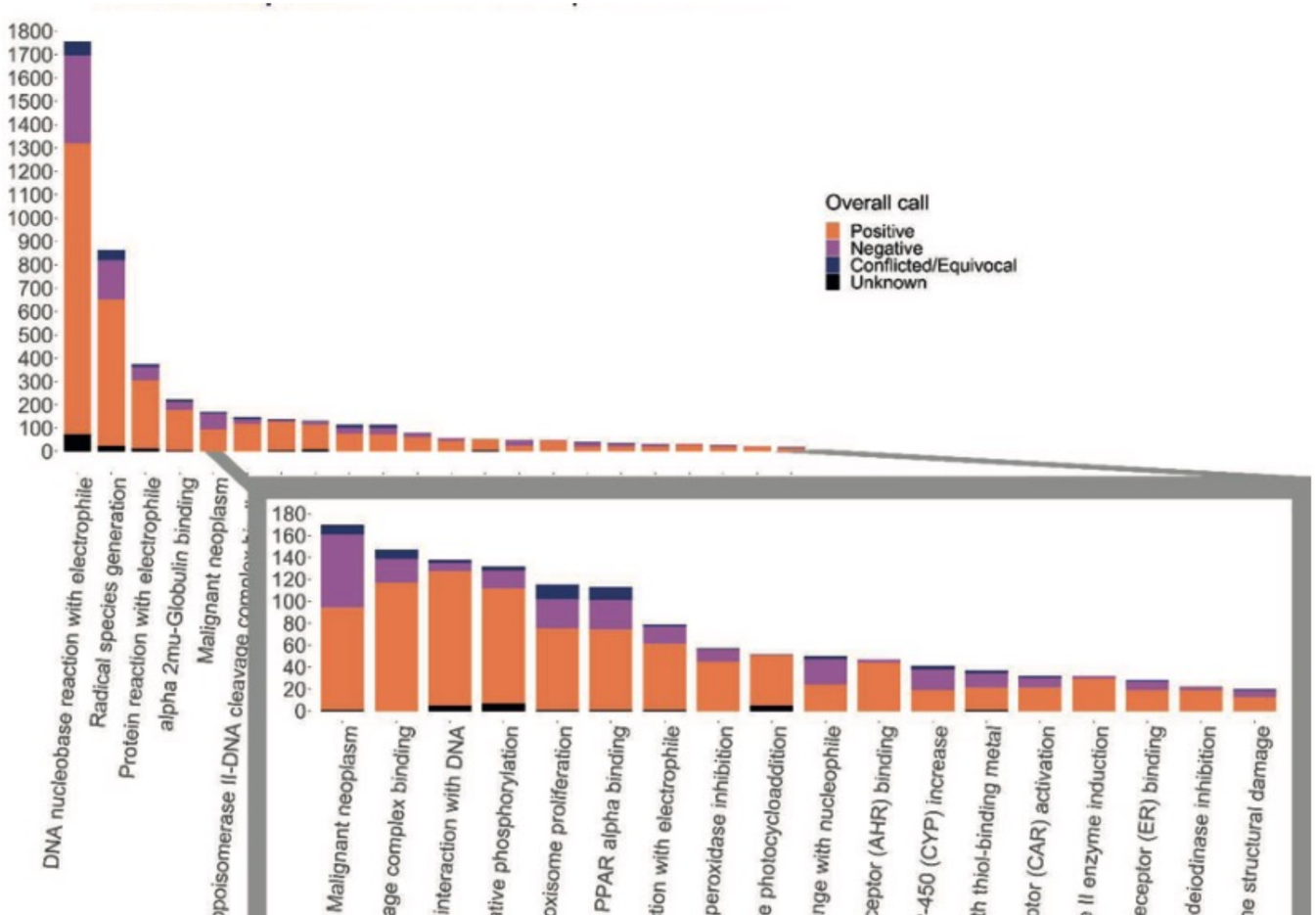
- Pharmaceuticals, agrochemicals, industrial chemicals, UVCBs, nanomaterials, polymers, LNPs, oligos

Gaps for Detecting Carcinogens

- Presently qualified to detect genotoxicants – covers genotoxic carcinogens
- Requires further investigation for carcinogens acting by other MIEs

Section 3: Important Key Events (KEs) in Carcinogenesis

- Over 13,000 chemicals used to draft an AOP based on rodent carcinogens (Cayley et al, 2023)



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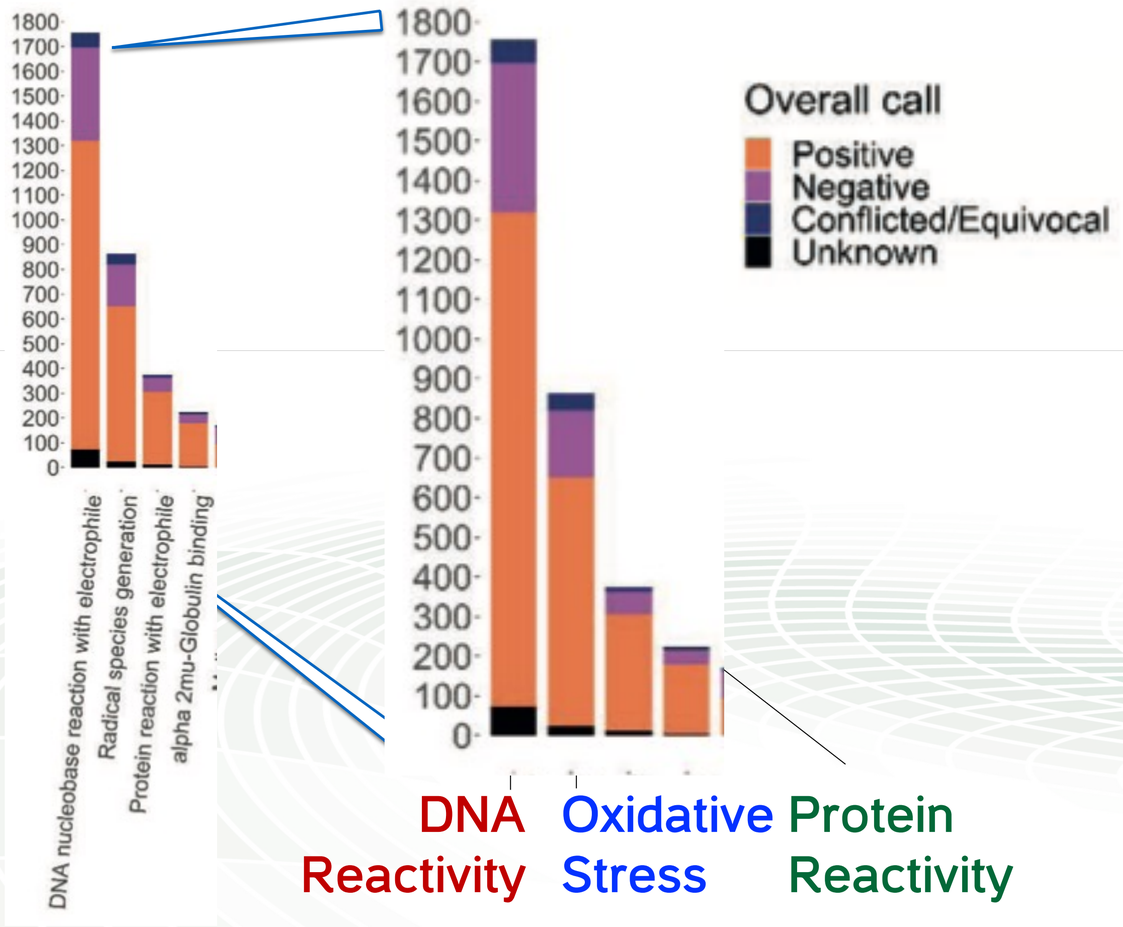

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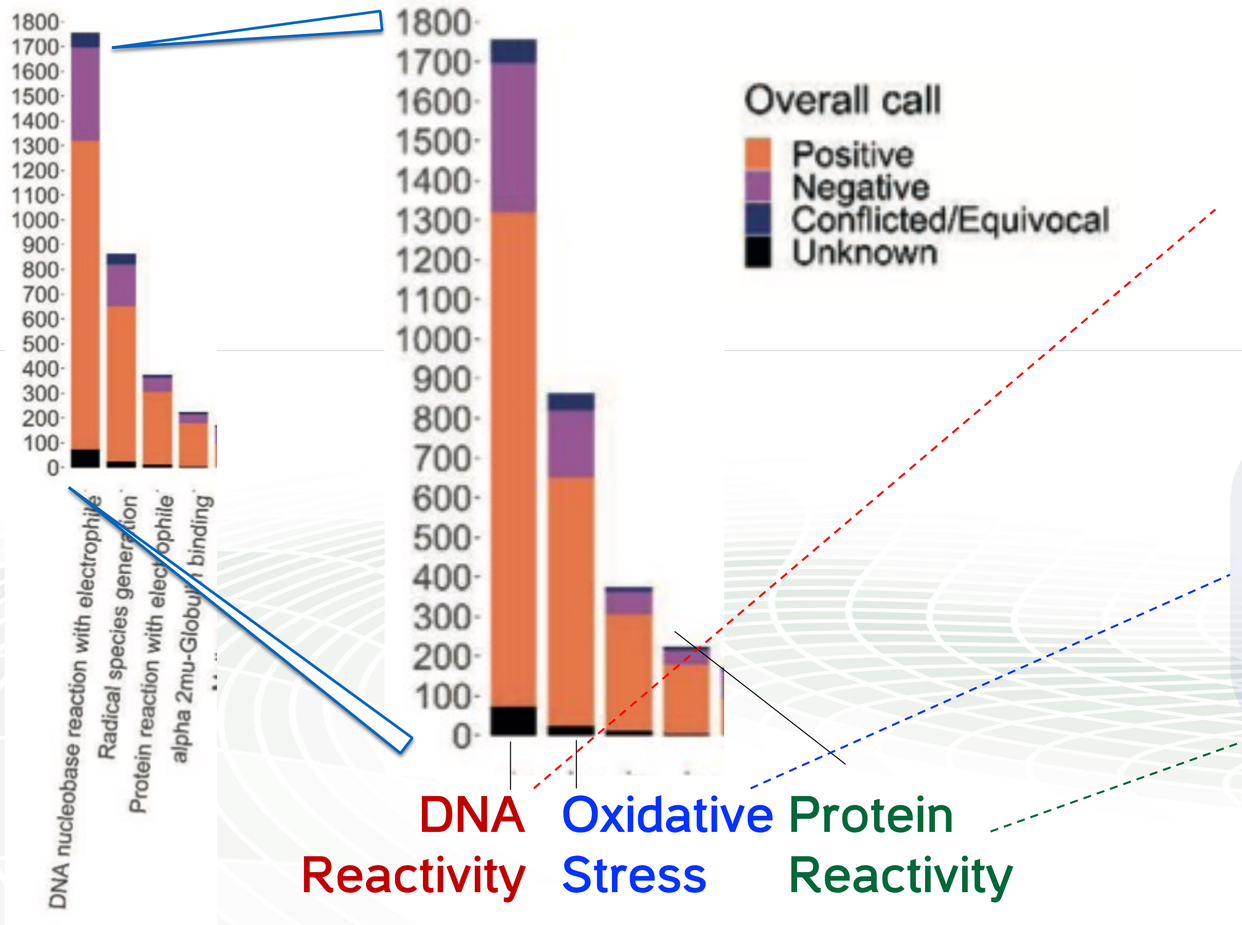

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Srxn1-GFP **Blvrb-GFP**

- Oxidative stress
- ROS production
- Indirect genotoxicity (AO)

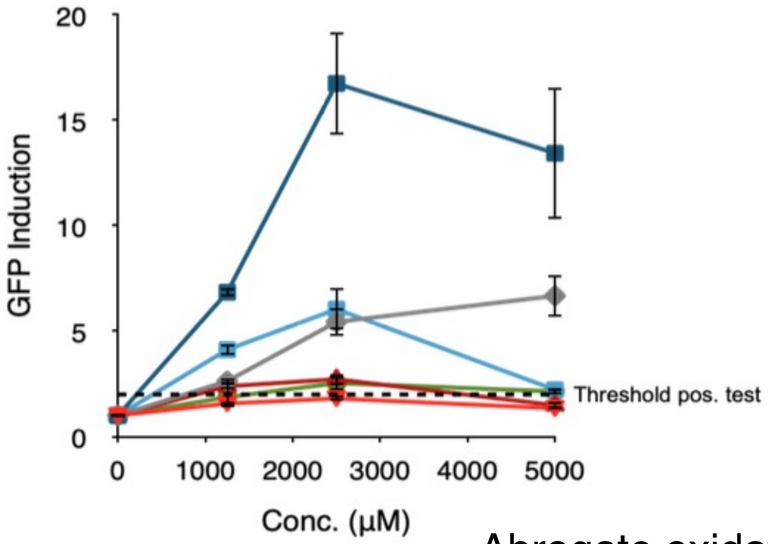
Ddit3-GFP

- Unfolded protein response
- ER stress

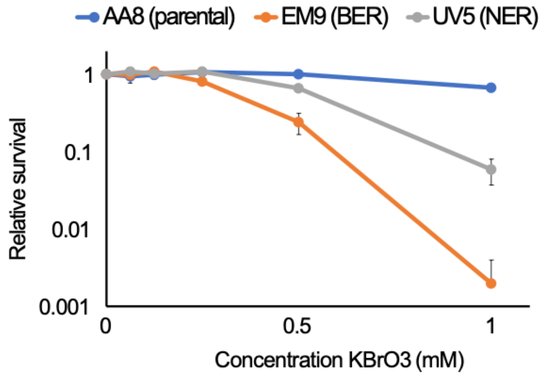
These pathways are the focus of ToxTracker

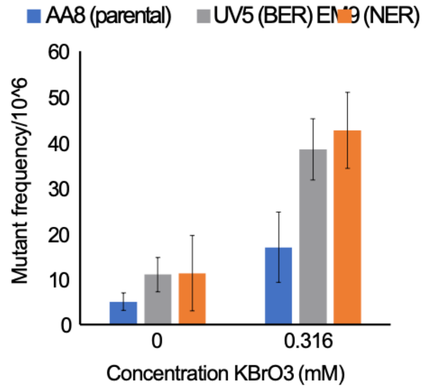
Section 3: Example Oxidant $KBrO_3$

Start with ToxTracker

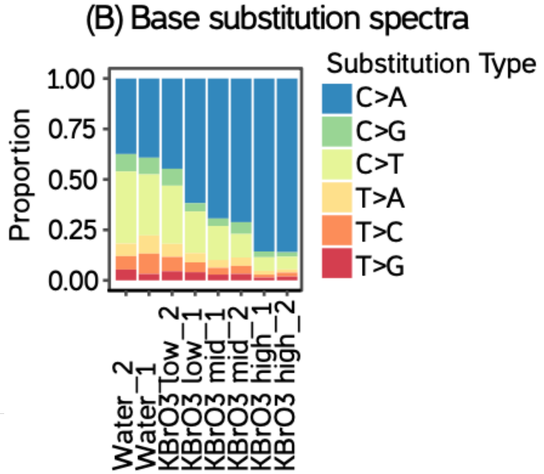


Repair deficient cell lines are more sensitive to 8oxoG lesions supporting the hypothesis….

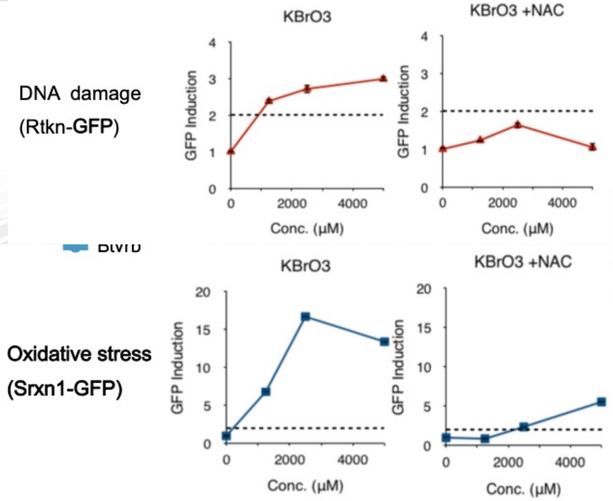


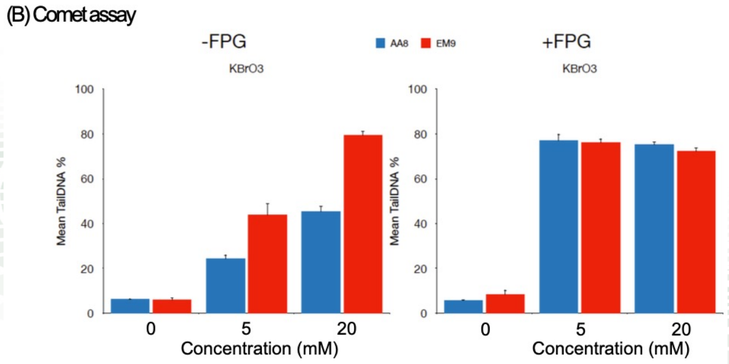


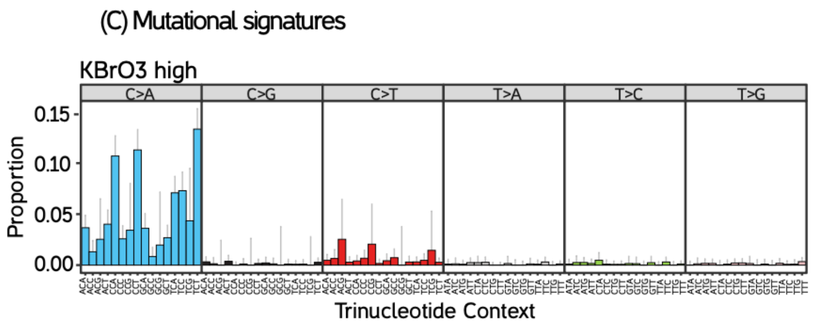
ecNGS to confirm



Abrogate oxidative DNA damage

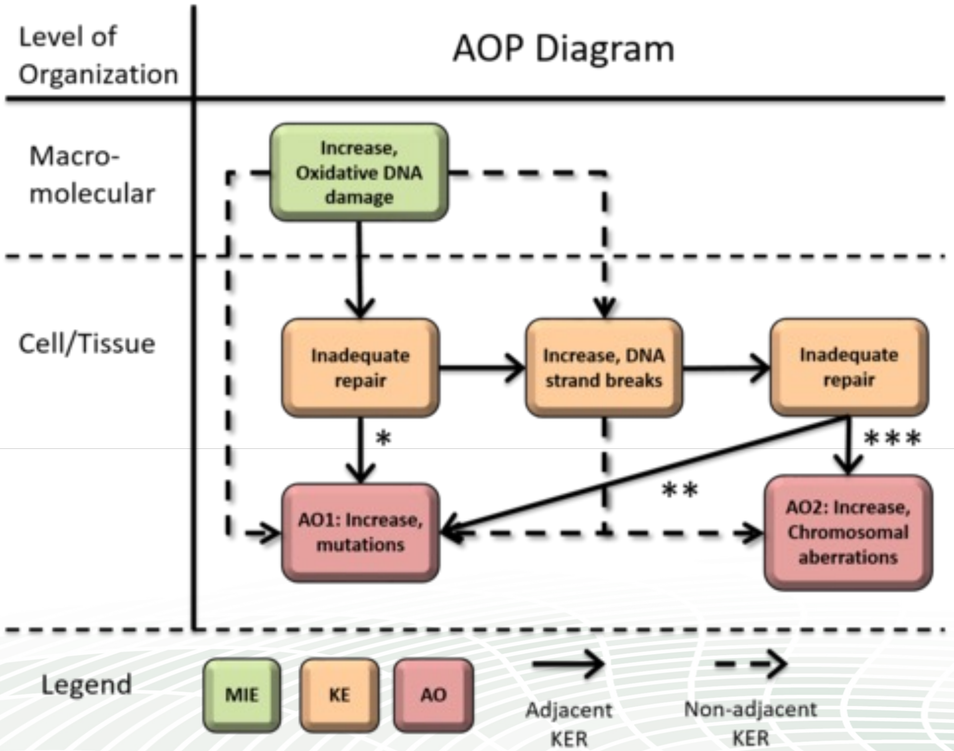




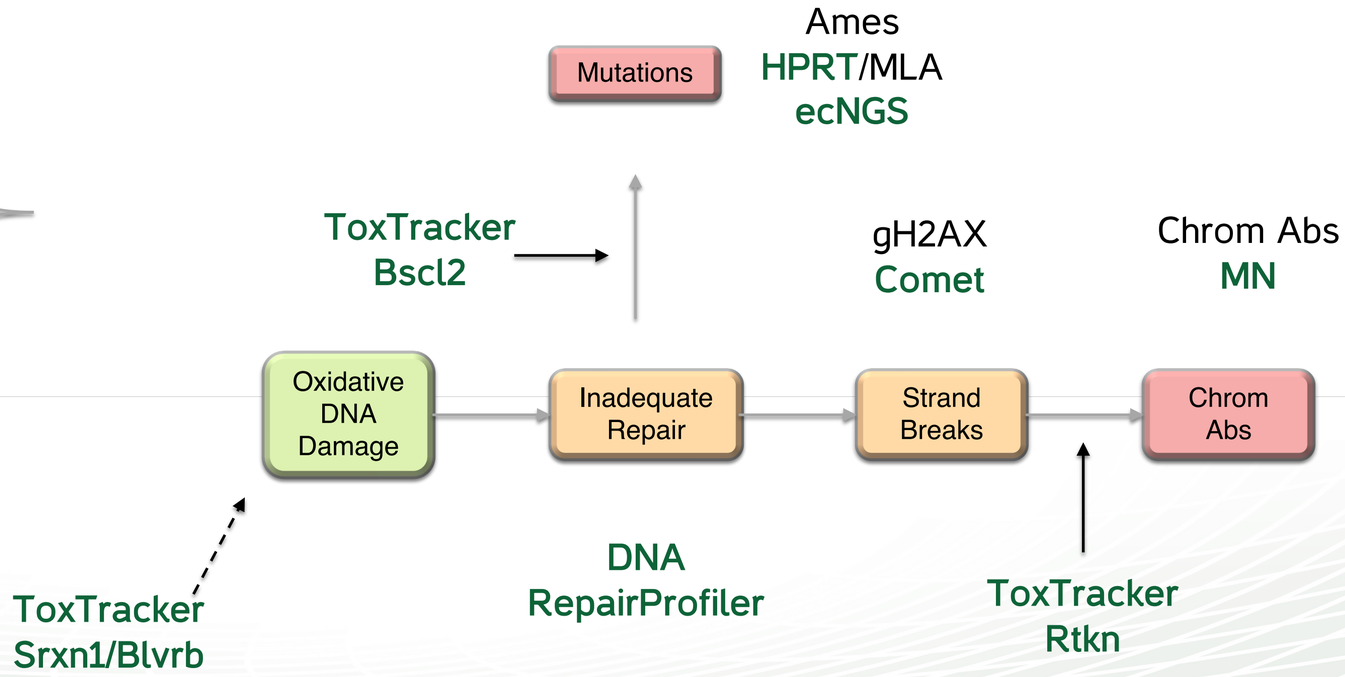


Mechanistic information to support AOPs

- AOP published on AOP wiki (https://aopwiki.org)
- DNA damage leading to chromosomal aberrations



*** Relevant repair pathway for Event 155: Excision repair**
**** Relevant repair pathway for Event 155: Error-prone double strand break repair (NHEJ)**
***** Relevant repair pathway for Event 155: Error-prone double strand break repair (NHEJ)**
"Inadequate DNA repair" (KE 155) is shown twice in this flow diagram to emphasize that "Increase, DNA strand breaks" (KE 1635) is required for the progression to AO2 "Increase, Chromosomal aberrations" (KE 1636), and that different repair pathways are involved in repairing different types of DNA damage.



Section 3: Qualification of the ToxTracker assay

- Thousands of compounds tested so far
- **Initially validated with Toxcast DB* and ECVAM-suggested** libraries**
- Interlaboratory OECD ring trial confirmed superior single assay sensitivity and specificity

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

* https://www.epa.gov/chemical-research/toxcast-chemicals
** Kirkland et al., 2016

Section 3: External Validation

International OECD inter-laboratory validation

- Extended 7 lab validation study w/ 64 compounds (2017-2022)
- Chemical selection was sector agnostic, ~half genotoxicants
- Conducted in accordance with OECD guidance document 34
- Expert Validation Management Team (VMT)
- Currently drafting a test guideline for WNT review

VMT Members	Industry	Location
David Kirkland	Kirkland consulting	UK
Philippe Vanparys	Gentoxicon	BE
Jan van Benthem	RIVM	NL
Els Adriaens	Adriaens consulting	BE
Giel Hendriks	Toxys	NL

BETTER POLICIES FOR BETTER LIVES

Participating Laboratories
Pfizer (US)
Genentech (US)
Roche (EU)
Procter & Gamble (US)
Corteva agriscience (US)
Charles River (CA)
Covance (EU)

* Sensitivity indicates the number of carcinogens that are positive in a genotoxicity test, the specificity indicated the percentage of non-carcinogens that give a negative results in a genotoxicity test. Sensitivity refers to the number of false-negative tests. Specificity indicates the fraction of false-positive test results.

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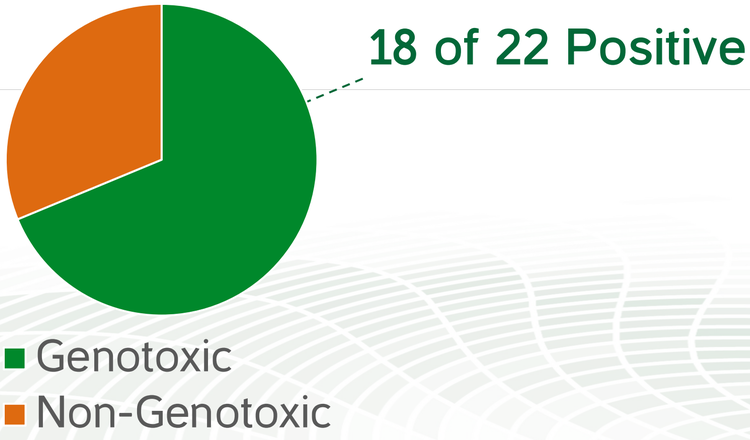
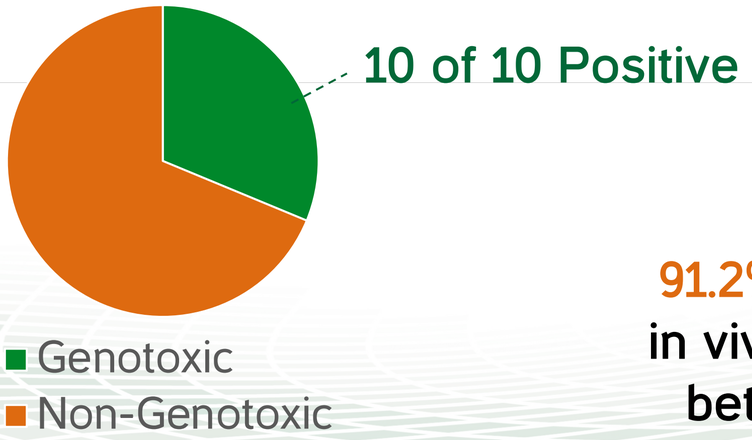
Carcinogens (n=32)



Non-Carcinogens (n=32)

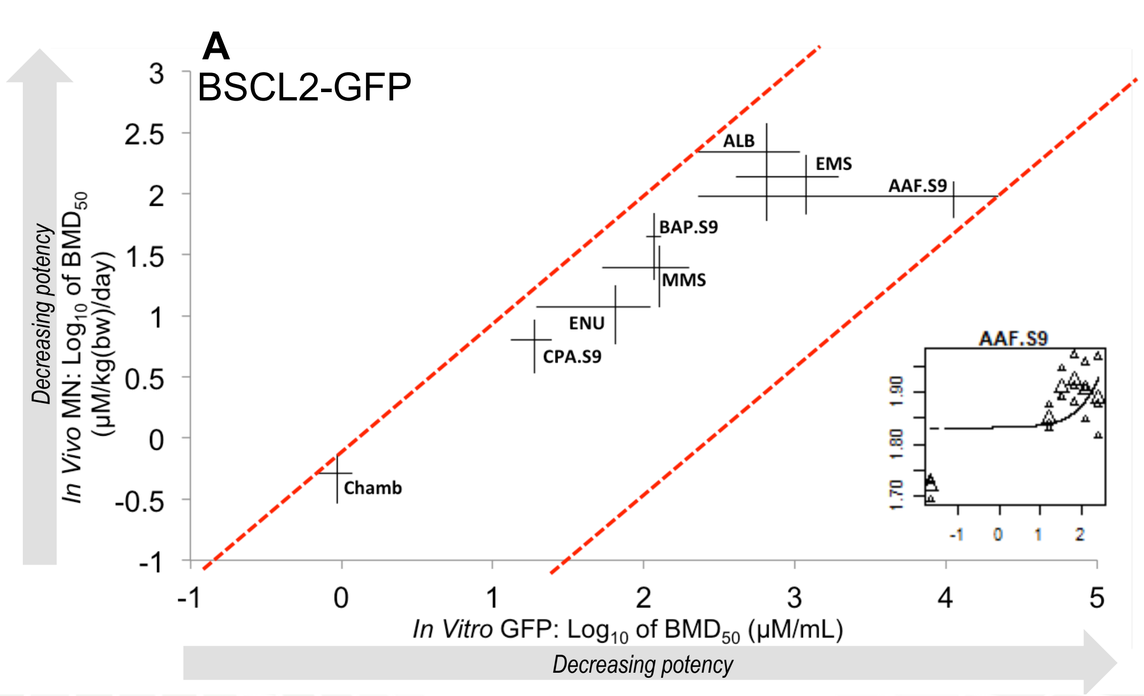


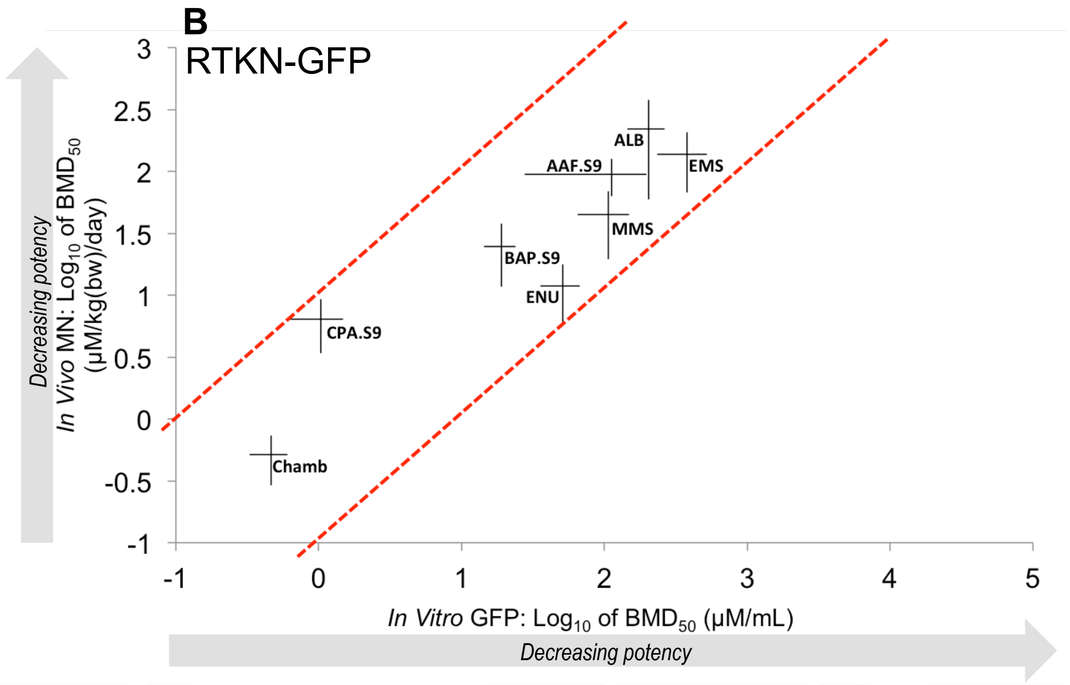
84.4% Sensitivity and 91.2% specificity for detecting in vivo genotoxicants with 83% between lab reproducibility
(Only Rtkn/Bscl2 Evaluated)

* https://www.epa.gov/chemical-research/toxcast-chemicals
** Kirkland et al., 2016

Section 3: BMD-based correlation between ToxTracker and in vivo MN

- Bscl2-GFP and Rtkn-GFP BMDs overlap with those obtained from in vivo MN studies
- This has potential to replace the in vivo POD metrics, supporting the 3Rs





Wills JW *et al.* Empirical comparison of genotoxic potency estimations: the *in vitro* DNA-damage ToxTracker endpoints versus the *in vivo* micronucleus assay. Mutagenesis. 2021 Aug 27;36(4):311-320.

Section 4: Assay Variability

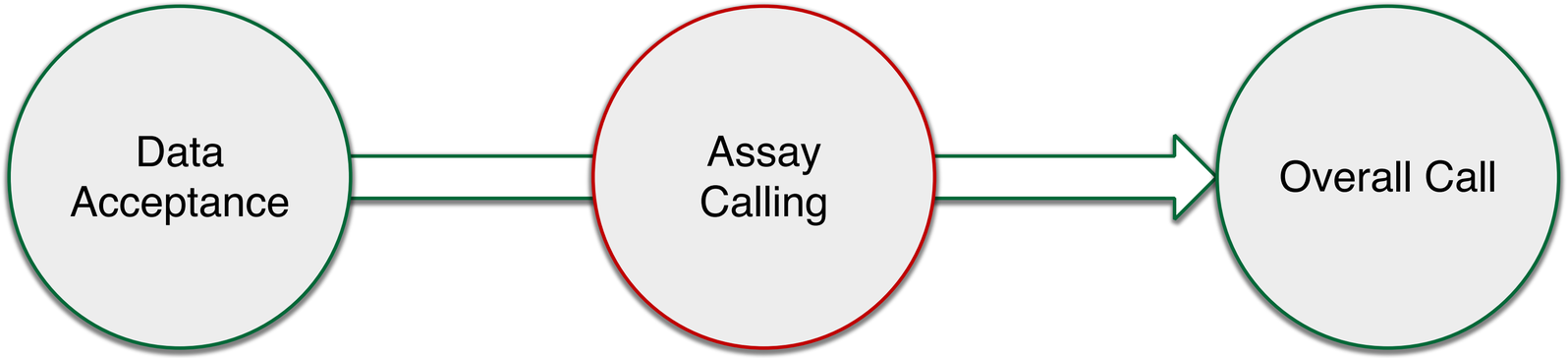
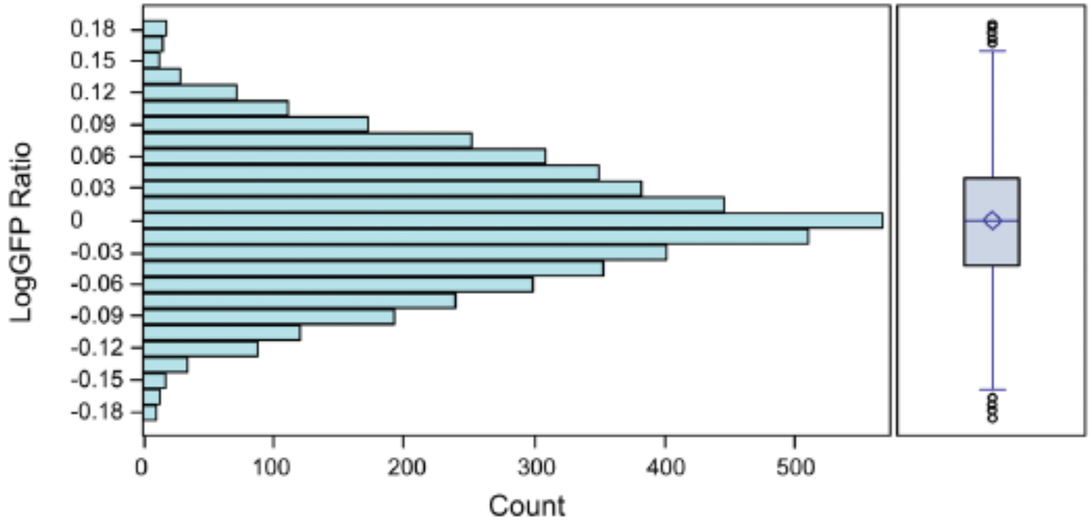


FIGURE 1 Illustration of a SAS bootstrap output for a single study. Control ratios were calculated 5000 times; the distribution of Log10 bootstrapped ratios was examined and compared to expectations for a normal distribution (e.g., histogram and box plot). The geometric 95th and 99th percentiles were used to determine the cut-off values for determination of a positive response (Table 1). LogGFPRatio = Log10 GFP Ratio.



Assessed variability in vehicle controls from >100 studies using bootstrapping techniques (5000 iterations, log-transformed data)

Section 4: Robust Response Criteria

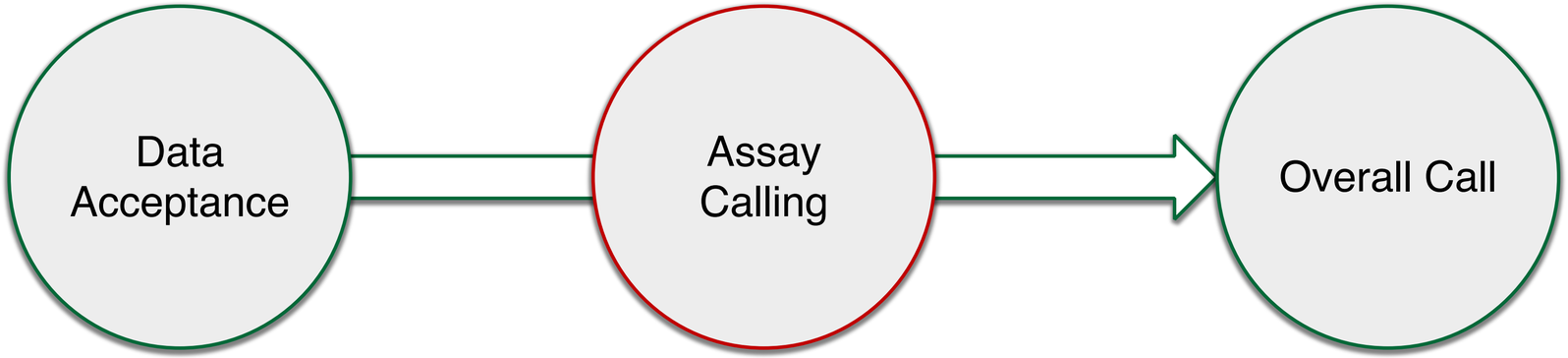


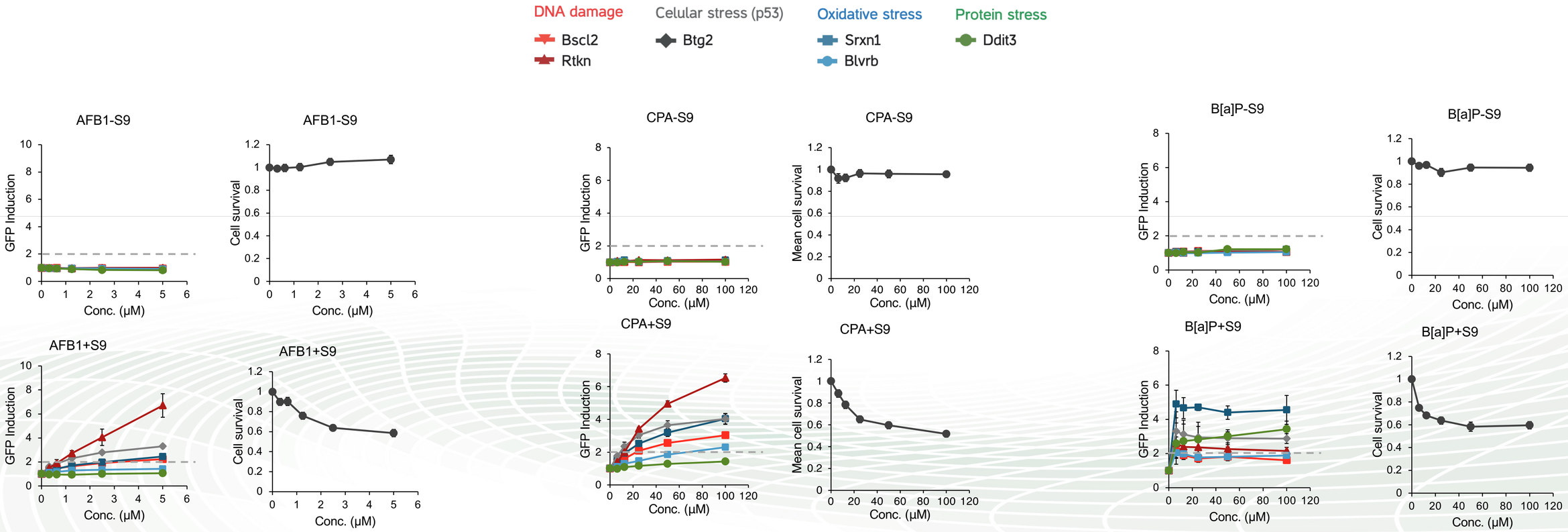
TABLE 1 Summary of bootstrap analysis to determine fold-change cut-off values for delineation of significant positive responses. Values shown are geometric 95th (GM95th) and 99th percentiles (GM99th) of ratio distributions.

Reporter	GM 95th	GM 99th
Bscl2	1.46	1.66
Rtkn	1.55	1.76
Btg2	1.46	1.64
Srxn1	1.58	1.85
Blvrb	1.62	1.88
Ddit3	1.52	1.69
Average	1.51	1.74

- Negative (< 1.5-fold induction)
- Equivocal (1.5 to < 2.0-fold induction)
- Positive (≥ 2.0-fold induction)

Section 4: Consistent culturing practices

- Metabolic activation of compounds by PB/Nf induced rat liver S9
- Same exposure paradigm used - absence or presence of 0.4% S9, for 24 hours.
- Facilitates direct comparison of treatment conditions within the assay



Section 4: Intra-laboratory reproducibility

- The within-laboratory reproducibility (WLR) was up to 98% (73%-98% across participants) and the overall between-laboratory reproducibility (BLR) was 83%.
- Evaluating just Rtkn and Bscl2, using consensus calling and the standard ± S9 protocol
- So far transferability is high, as the assay is conducted in multiple other labs worldwide

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%

Concluding SWOT for detecting carcinogens

Strengths

- Qualified/validated for detecting direct and indirect genotoxicants
- High throughput, simple gating logic, and easily transferred to other laboratories.
- Used for hazard ID with data supporting quantitative risk assessment processes

Weaknesses

- Other cell lines (Srxn1, Blvrb and Ddit3) have not been extensively evaluated despite being selected for their uniqueness in discerning carcinogens from non-carcinogens.

Opportunity

- To create and test a library focused on the 3 major KEs in Cayley et al (2023) and adequately assess the other cell lines for use in carcinogenicity testing.

Threats

- Genomics technologies for identifying direct genotoxicants (i.e., ecNGS)
- Other genetox NAMs

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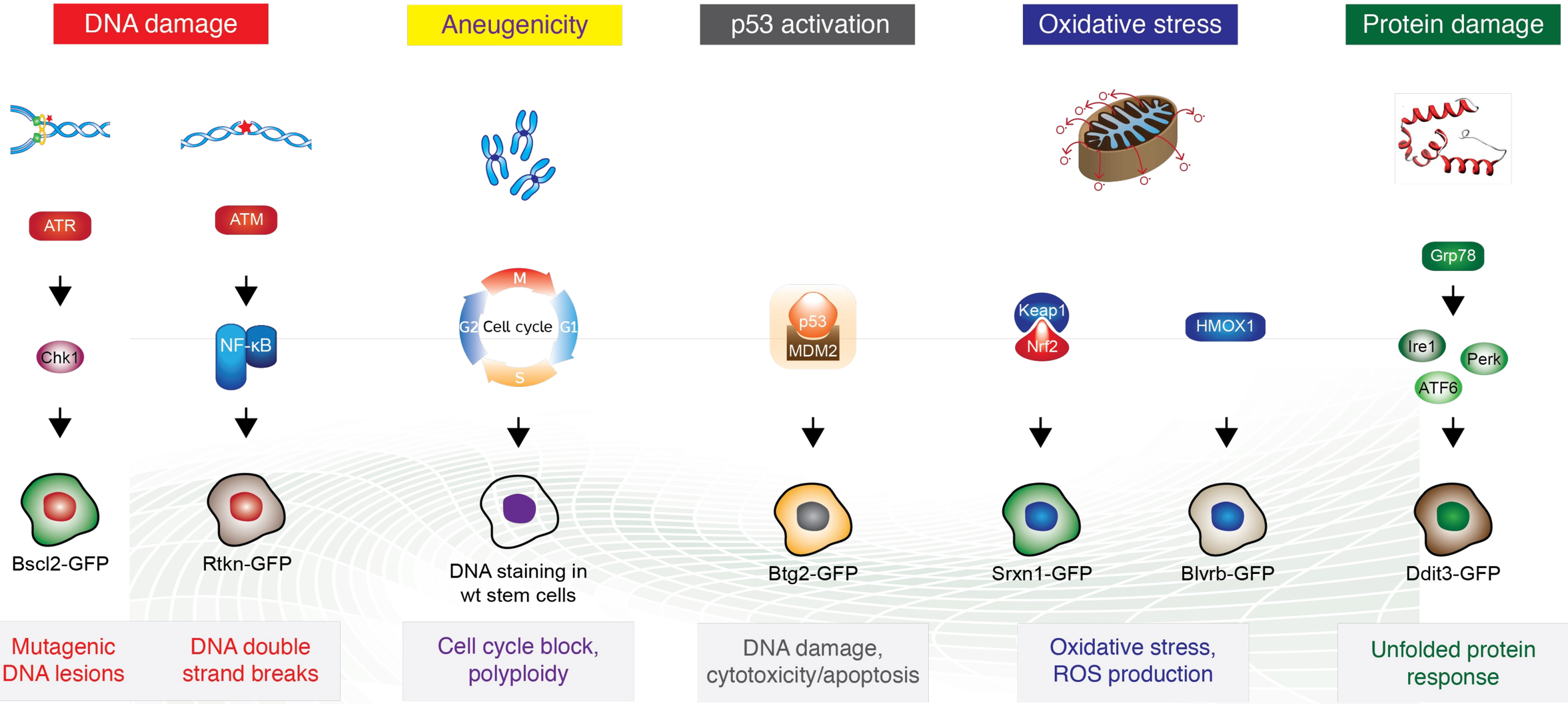
Back up slides

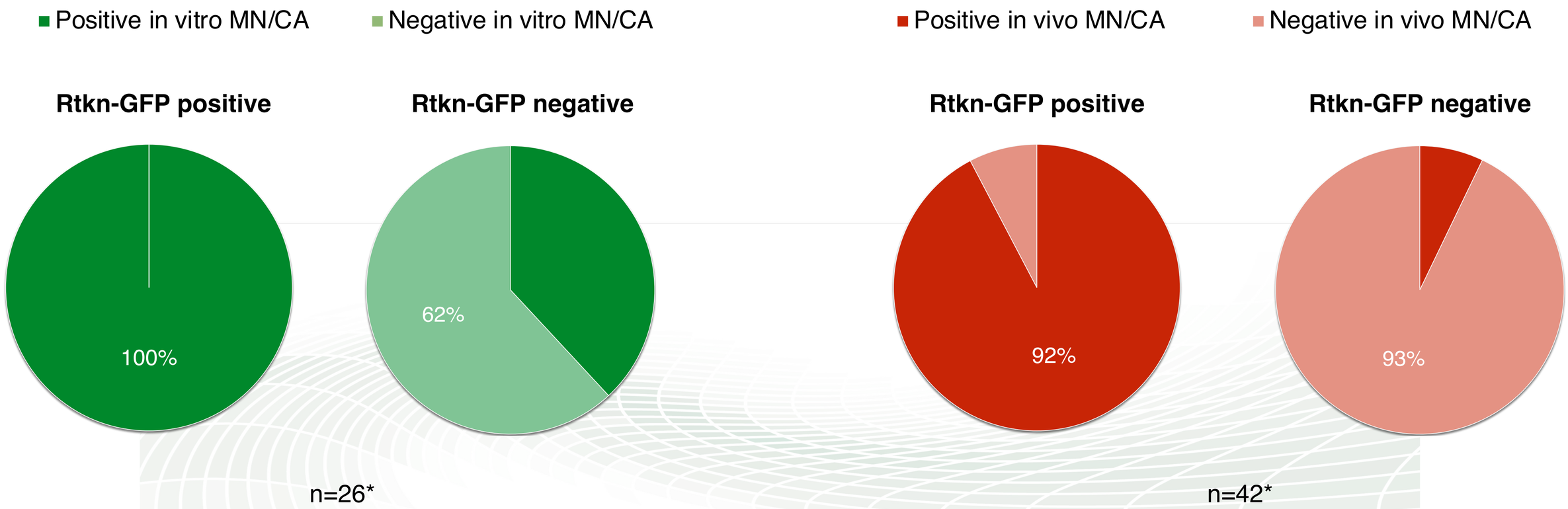
Refresher: Biological Coverage

- ToxTrackerACE integrates cell cycle analysis into the ToxTracker assay to discern aneugens



ToxTracker correlates with regulatory genetox assays

- Rtkn-GFP reporter for DNA strand breaks is predictive for micronucleus (MN) and chromosome aberration (CA) assays



*Comparison with ECVAM library of reference compounds, Kirkland et al 2016

Genotoxic MoA investigation in ToxTracker

	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

Blue: expected MoA oxidative stress

Green: expected MoA protein unfolding