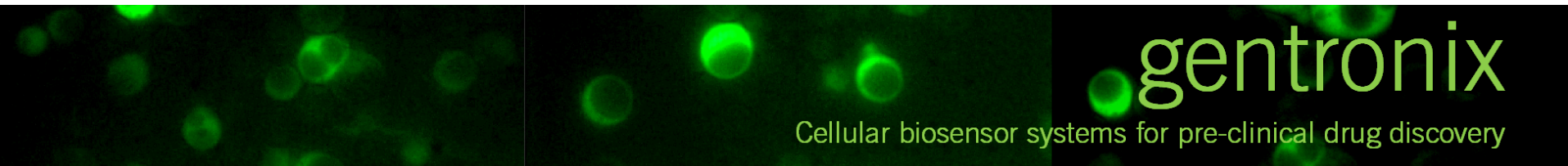


The TK6 Gadd45a-GFP Genotoxicity assay



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Cellular biosensor systems for pre-clinical drug discovery

The problem with genetic toxicology,

Data from Kirkland et. al., 2005

a new solution: the Gadd45a assay,

Data from Hastwell et. al. 2006 GSK/Gentronix

and some new assay data

Richard Walmsley, CSO/Founder Gentronix Ltd

The logo for Gentronix, featuring the word "gentronix" in a lowercase, sans-serif font. The letters are white with a green glow effect, set against a dark background with a bokeh of green light spots.

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Scope for COM

The utility of the new assay in screening for mutagenicity and other forms of genome damage:

Validation of the GADD45a biomarker

Method, and appearance of results

Interpretation of the results

Rationale for using as a genotox hazard screen

Use of S-9 metabolic activation

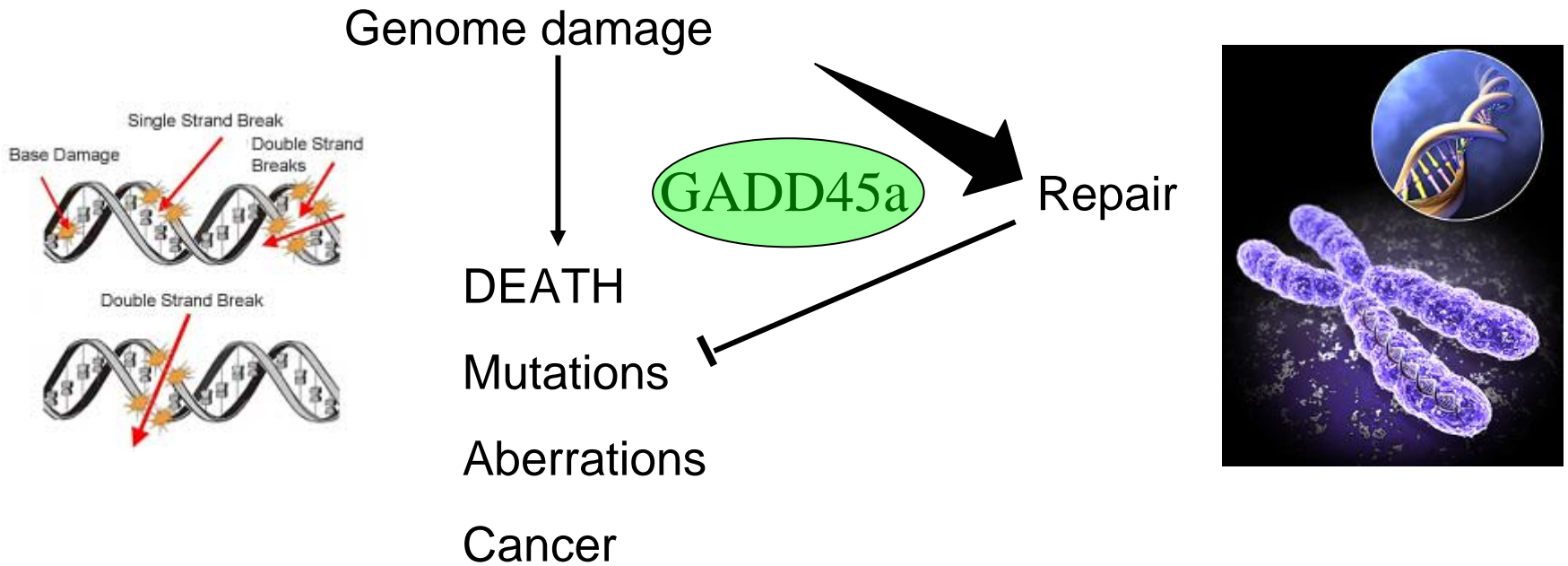
Likely use in the current screening context



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Context: the DNA damage hazard



**Genetic endpoints
(regulatory tests)**

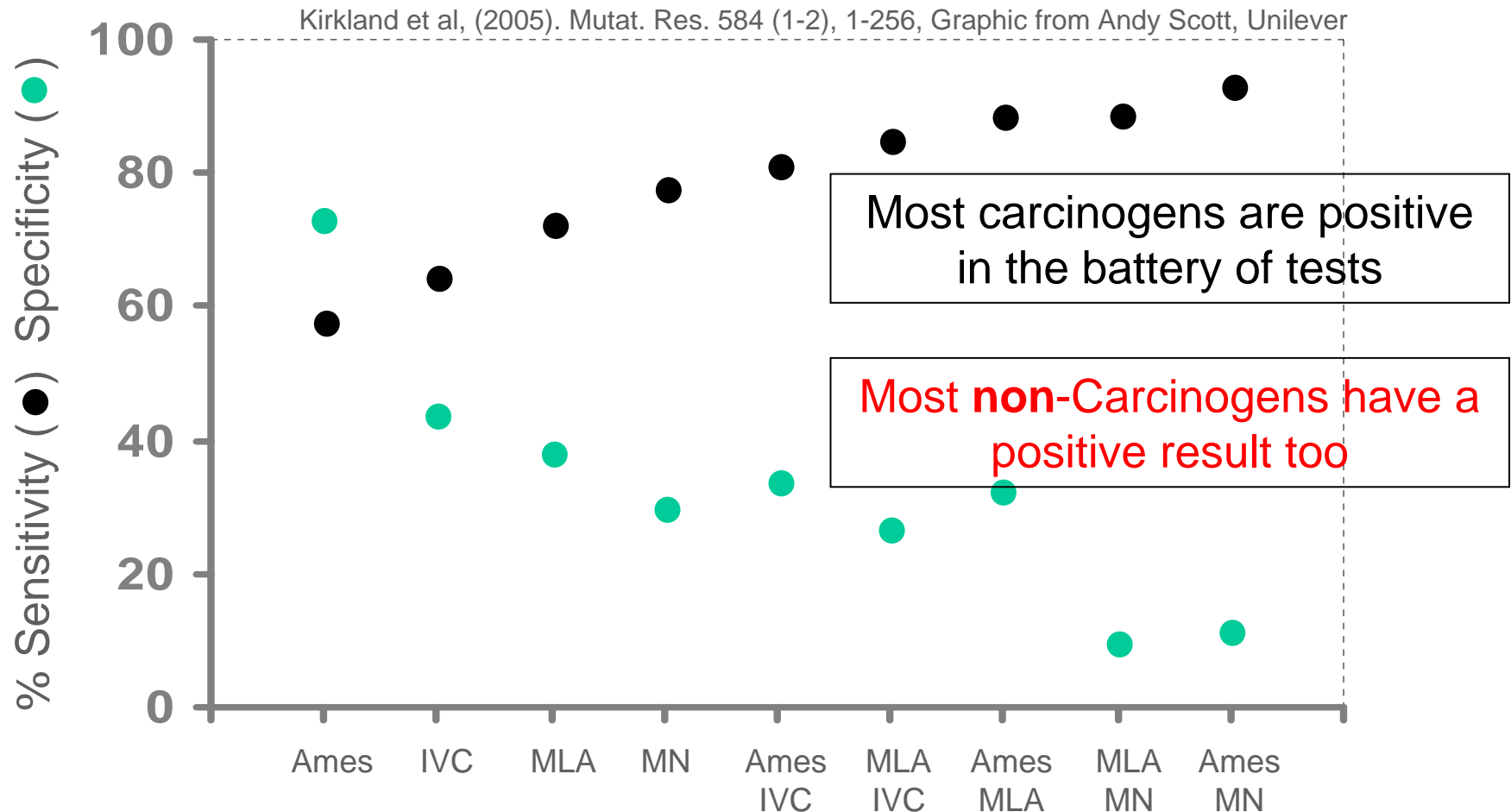
**Response endpoints
sensing or repairing
(Gentronix biomarkers)**

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in vitro genotox testing: the problem

...poor specificity



It is not just Kirkland *et al.*...

“The current FDA regulatory battery of four genotox tests used to predict carcinogenicity includes two tests with good correlation (gene mutation in Salmonella and *in vivo* micronucleus) and two tests with poor correlation (mouse lymphoma gene mutation and *in vitro* chrom. abs.)”

Matthews, E.J. et al., 2006 Reg. Tox. Pharma. 44 83-96



35% of submitted pharmaceutical compounds are *in vitro* positive. *Peter Kasper*

15% of non-carcinogens are positive in chrom. abs
Snyder and Green, 2001, Mut. Res, 488, 151-169

All studies probably underestimate chemical space, as many more candidates will have been rejected before reaching submission.

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The consequences of the problem?

“need to reduce false positive results with *in vitro* genotoxicity testing and avoid unnecessary follow-up animal tests”

ECVAM , 26-28 April 2006”



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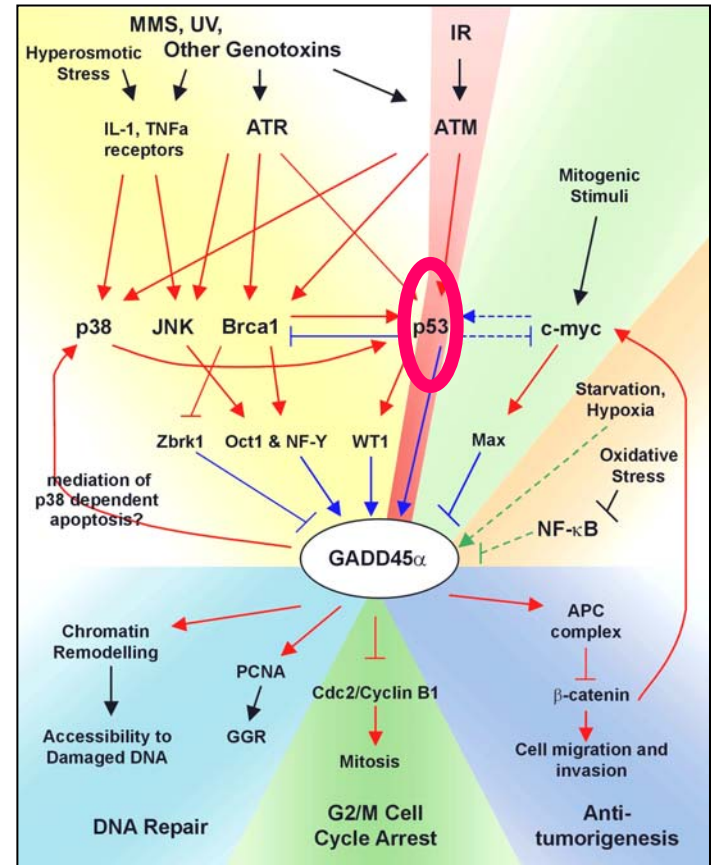
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A solution to the problem

GADD45a is well-characterised and has a central role in genomic integrity. It was discovered and named by Al Fornace in 1988

Genotoxic stress induces GADD45a transcription – adaptive response

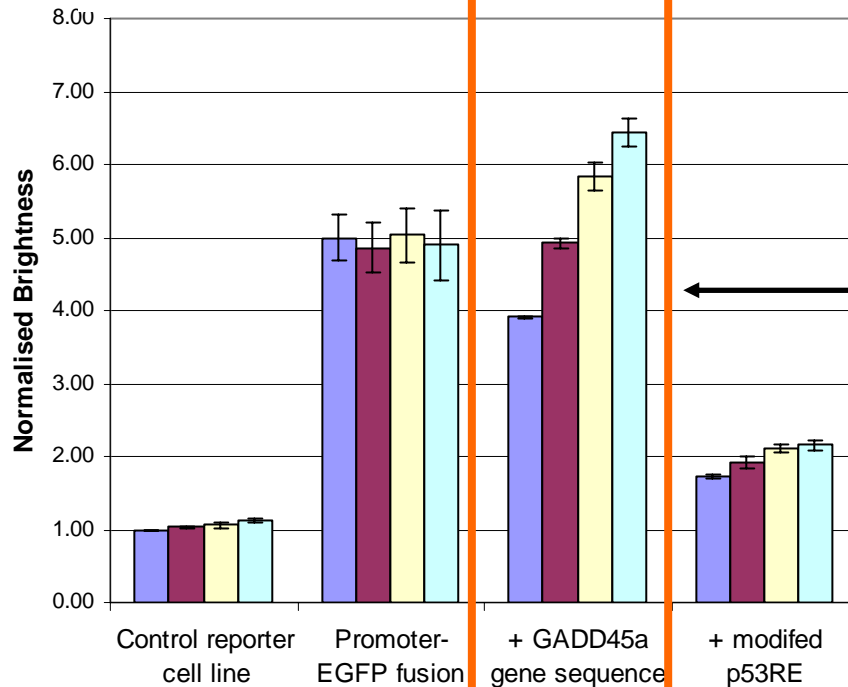
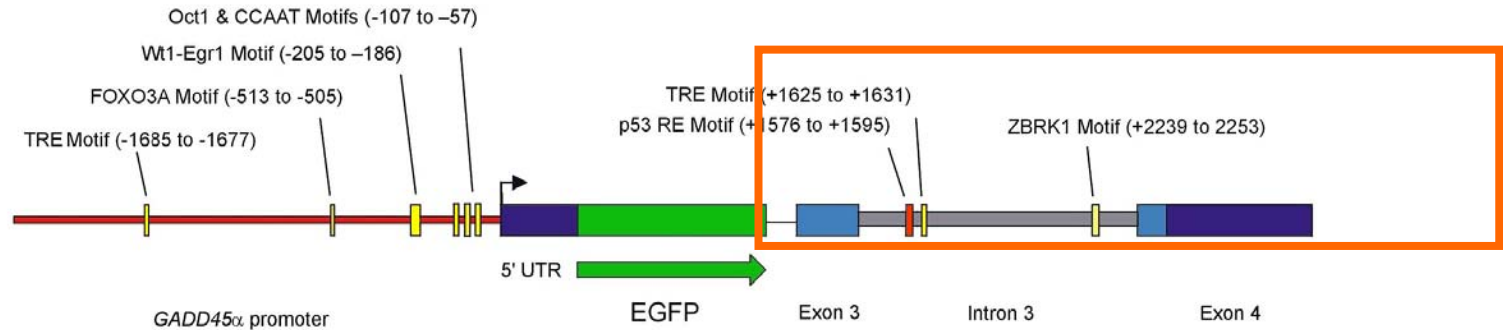
A new reporter has been constructed that exploits p53-dependent, genotoxin specific induction of human GADD45a expression in TK6 cells



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GADD45a Reporter Development

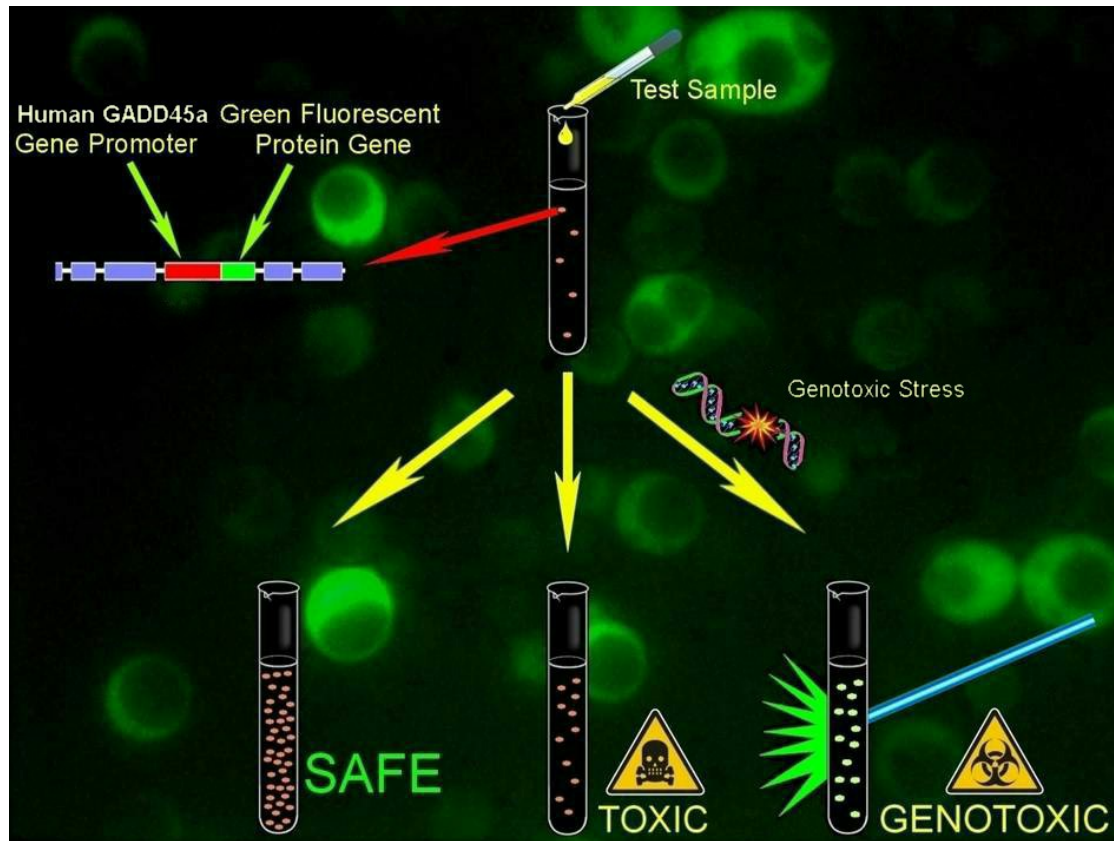


Promoter fusion is not sufficient for proper response. It needs the p53 response element from intron 3

0 µg/ml Cisplatin, 1 µg/ml Cisplatin, 2 µg/ml Cisplatin, 4 µg/ml Cisplatin

Assay principle:

DNA damage increases cellular fluorescence



Mix



Wait

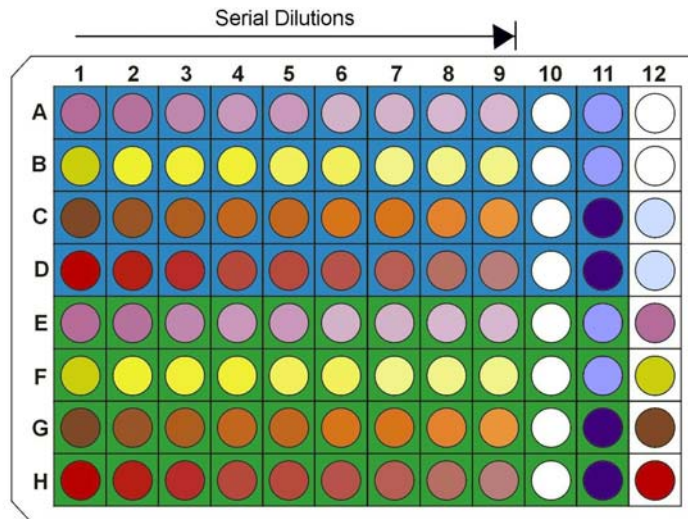


Measure

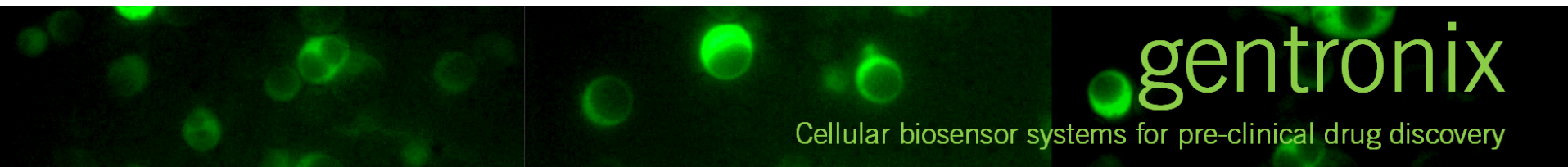
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Assay Overview



- 4 compounds per plate
- 9 two-fold dilutions
- Internal positive controls
- Test control – no GFP
- Plate set up in 20 minutes
- Results in 24/48 hours
- 20/day, 60/week (manual)
- Many more robotically
- 72/day, 216/week (3 concs)
- 1mg tests up to 1000 $\mu\text{g/ml}$
- 10ul of 10mM=100 μM



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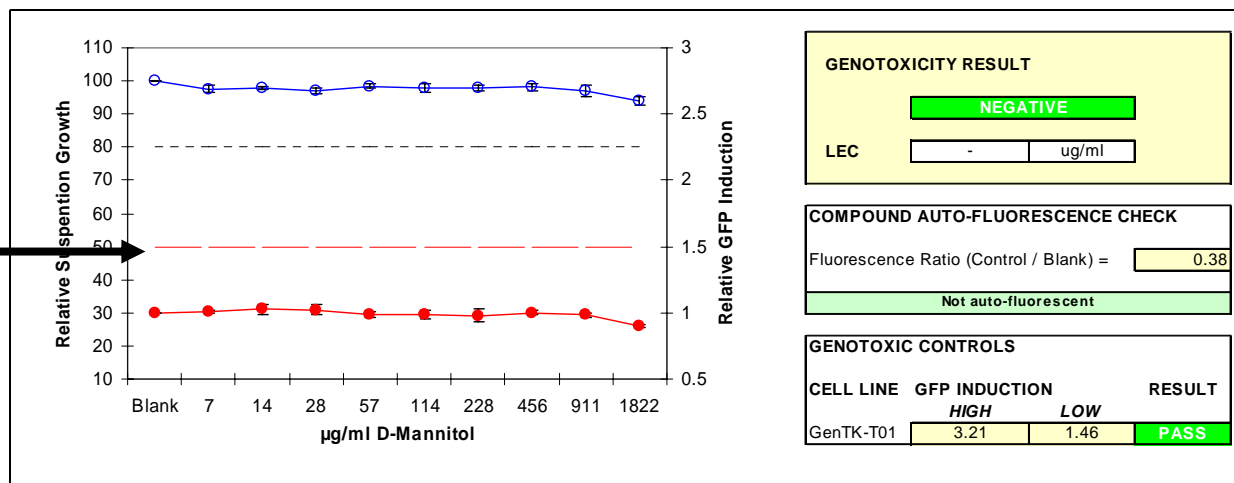
Cellular biosensor systems for pre-clinical drug discovery

Results – simple graphical output

Positive for genotoxicity if relative **fluorescence induction** exceeds threshold - - - -

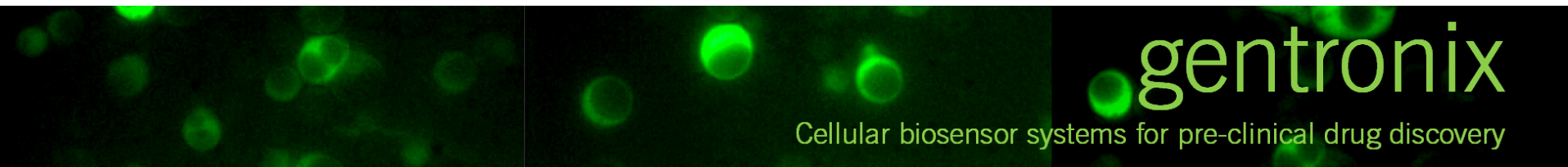
Negative result

Red line does not cross threshold



Genotoxicity threshold at 1.5 relative GFP induction:

- 3 x standard deviation in untreated samples
- data demonstrates biological relevance
- assay development might lower threshold



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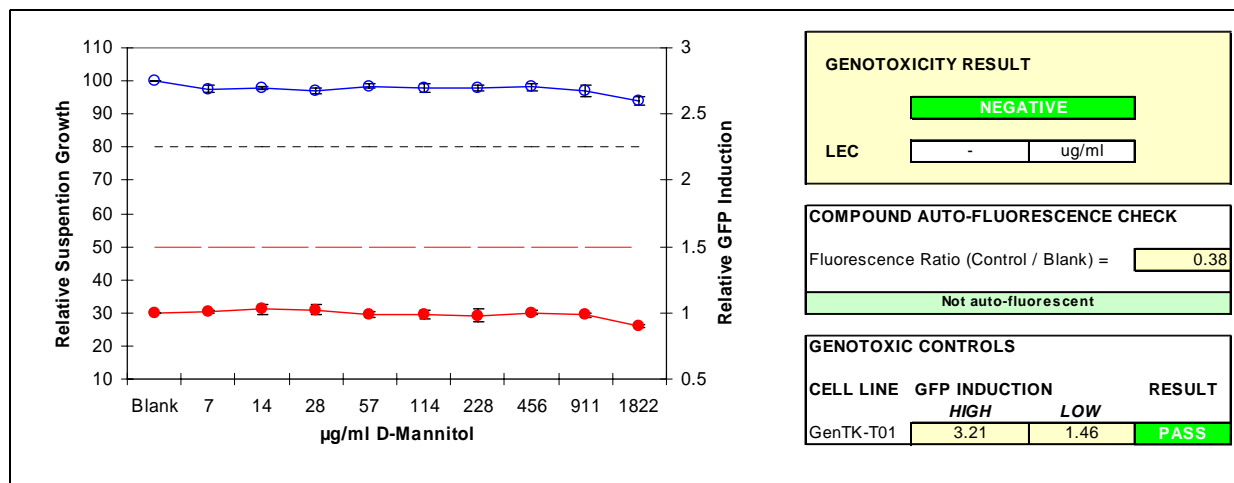
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Results – simple graphical output

Positive for genotoxicity if relative **fluorescence induction** exceeds 1.5 threshold - - - -

Negative result

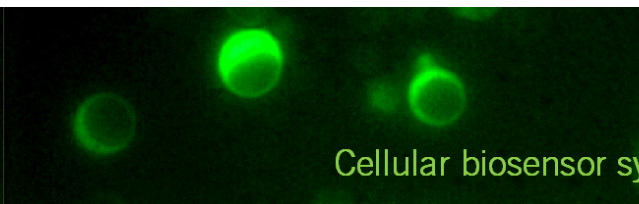
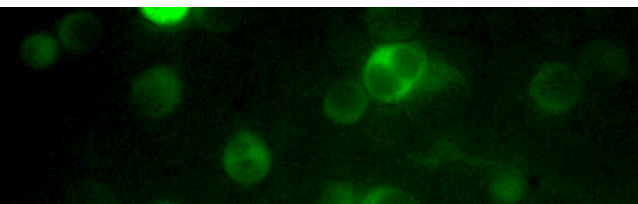
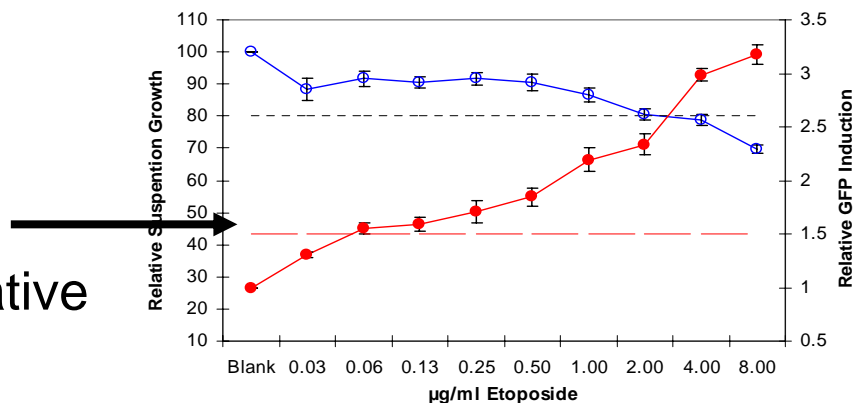
Red line does not cross threshold



Positive result

Red Line crosses threshold

Blue line shows relative suspension growth



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Notes on toxicity and RSG

ICCVAM, NICEATM, 2001

2.2 Recommended Measurement Endpoints for Basal Cytotoxicity.

1) **Inhibition of cell proliferation**: Cell number, Cell protein, DNA content, DNA synthesis, Colony formation...

Gentronix GreenScreen HC:

- The **inhibition of cell proliferation** in exposed cells w.r.t. unexposed cells is estimated by OD, and used to calculate Relative Suspension Growth
- The primary use of the OD data is normalisation of fluorescence data to produce a brightness value (Flu/OD), distinguishing few bright cells from many dim cells
- The software flags up 20% inhibition (80% RSG) as a cytotoxic effect. This is a small effect, and does not mean 20% of cells are dead. Indeed cells have all completed at least one cycle. It alerts the user to look at the data. 30% RSG inhibition means cells have not managed a cell division, and usually 90% dead. Remove alert?
- RSG is not supposed to indicate potential drug toxicity in humans
- Acutely low RSG only leads to increased brightness with genotoxins (data follows)
- Unexposed cells grow from (Tecan) OD= 0.02 to OD=0.07 over the 48h period

GFP is very stable.

The Brightness values taken at the 24/48h endpoint represent the integrated measure of all GFP produced during exposure. It is not a snapshot of repair activity at the end point.

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Mutation Research xxx (2006) xxx–xxx



Genetic Toxicology and
Environmental Mutagenesis

www.elsevier.com/locate/gen tox

Community address: www.elsevier.com/locate/mutres

High-specificity and high-sensitivity genotoxicity assessment in a human cell line: Validation of the GreenScreen HC *GADD45a-GFP* genotoxicity assay

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James S. Harvey^b, Robert W. Rees^b, Richard M. Walmsley^{a,c,*}

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Received 24 February 2006; received in revised form 4 April 2006; accepted 7 April 2006

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Published Validation (a)

does the assay identify genotoxins?

Tested 34 genotoxic agents, known mechanism of action, positive result in at least one ICH battery test.

- **10 Direct acting genotoxins**

4-Nitroquinoline-N-Oxide, Busulfan, Cisplatin, Ethyl methanesulfonate, Ethyl nitrosourea, ICR-191, Methyl nitrosourea, Mitomycin C, MMS, MNNG

- **10 Aneugens**

Colchicine, Demecolcine, Griseofulvin, Nocodazole, Paclitaxel, Thiabendazole, Vinblastine, Vincristine sulphate, Benomyl, Chloral hydrate

- **7 Nucleotide synthesis inhibitors**

5-Azacytidine, 5-Fluorouracil, Aphidicolin, Didanosine, Hydroxyurea, Pyrimethamine, Zidovudine

- **4 Topoisomerase inhibitors**

Amsacrine (m-AMSA), Camptothecin, Doxorubicin (Adriamycin), Etoposide

- **3 Reactive Oxygen Species**

Bleomycin sulfate, Hydrogen peroxide, Methyl viologen dichlorate

New: Sodium butyrate, a histone deacetylase inhibitor, it is positive

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Published Validation (b)

is the assay specific to genotoxins?

Tested 41 non-genotoxic agents – including ‘false positives’ from other tests

- **30 non-genotoxic agents with no positive *in vitro* genotoxicity data**

3-Amino-1,2,4-triazole, Acetonitrile, Ampicillin, Benzoin, Boric Acid, Caprolactam, Cyclohexanone, Dimethylformamide, D-Mannitol, DMSO, EDTA, trisodium salt, Ephedrine sulphate, Ethanol, Ethylene glycol, NN Dimethylurea, Phenformin hydrochloride, Phenylephrine hydrochloride, Propantheline Bromide, Pyrazine, Pyridine, Roxarsone, Sodium chloride, Sodium Dodecyl Sulfate, Sucrose, Tetracycline, Tetrahydrofuran, Titanium dioxide, Tritolyl phosphate, Urethane

- **11 cytotox positives – they have positive *in vitro* chromosome aberration data associated with cytotoxicity (Hilliard *et al.*, 1998), but are negative in other *in vitro* tests, and *in vivo* MNT/cancer (i.e. ‘false positive’ for *in vivo* hazard).**

2,4-Dichlorophenol, 3,5-Dichlorophenol, 2,4-Dinitrophenol, Bisphenol A, Chloramphenicol, D,L-Menthol, Dithiocarb, Ethionamide, Phthalic anhydride, Sodium iodoacetate, Sodium xylenesulfonate

- **All validation chemicals tested at least 4 times, to 10 mM or to limit of solubility or cytotoxicity, whichever is the lower.**

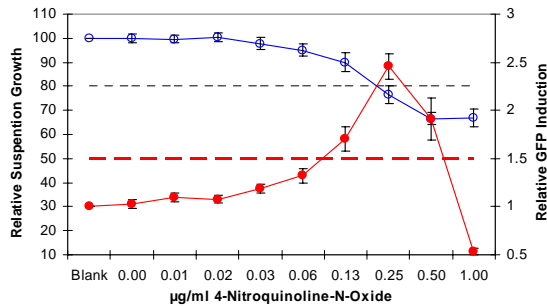
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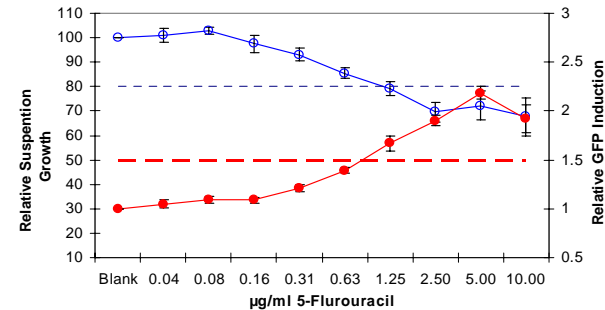
Genotoxins give positive results

- 31 of the 34 'genotoxic' agents robustly induced the GADD45a reporter. All mechanistic classes detected. **High sensitivity.**

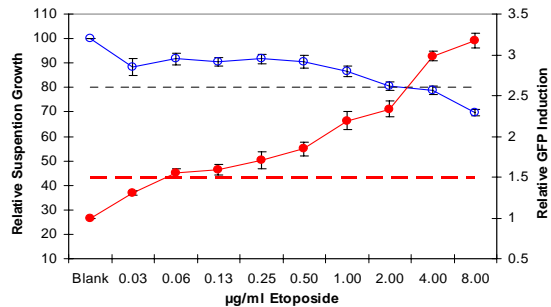
4-Nitroquinoline-N-Oxide (direct)



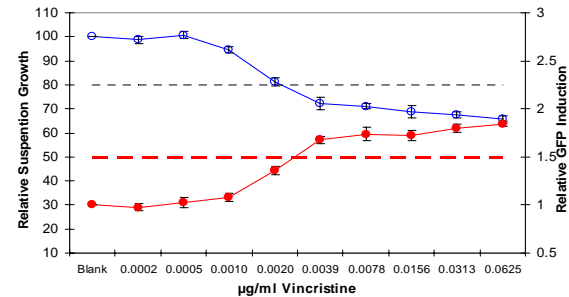
5-Flurouracil (nucleoside analogue)



Etoposide (topoisomerase II inhibitor)



Vincristine (aneugen)



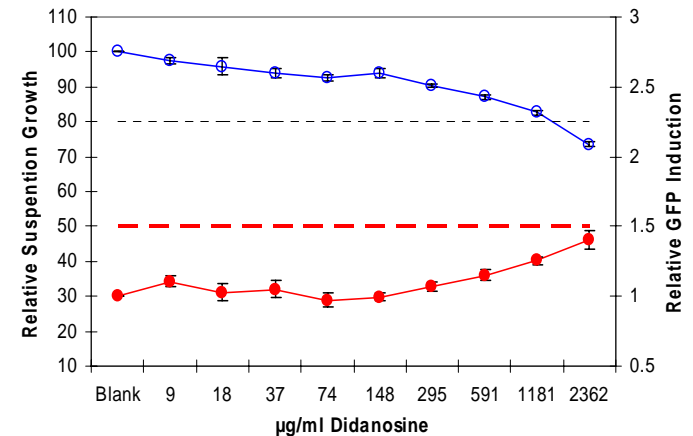
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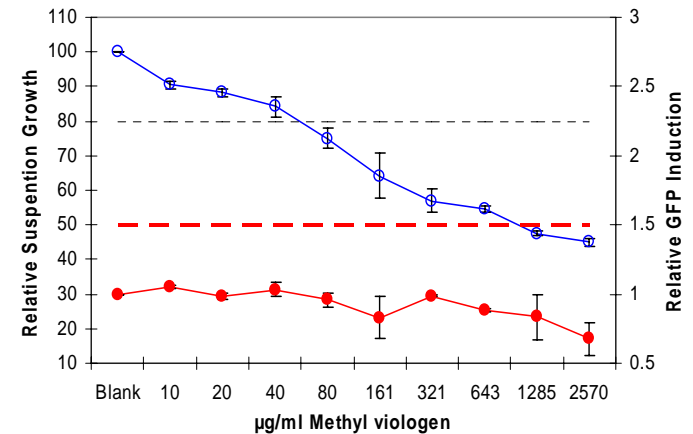
3 'genotoxins' give negative results

- Didanosine (a nucleoside analogue)
Equivocal regulatory data;
Equivocal GADD45 data : 2+ 2-
- Thiabendazole: (not an aneugen?)
Original MNT data not reproducible
- Methyl viologen (Paraquat, a ROS)
Did not induce GADD45a reporter.

Didanosine



Methyl viologen



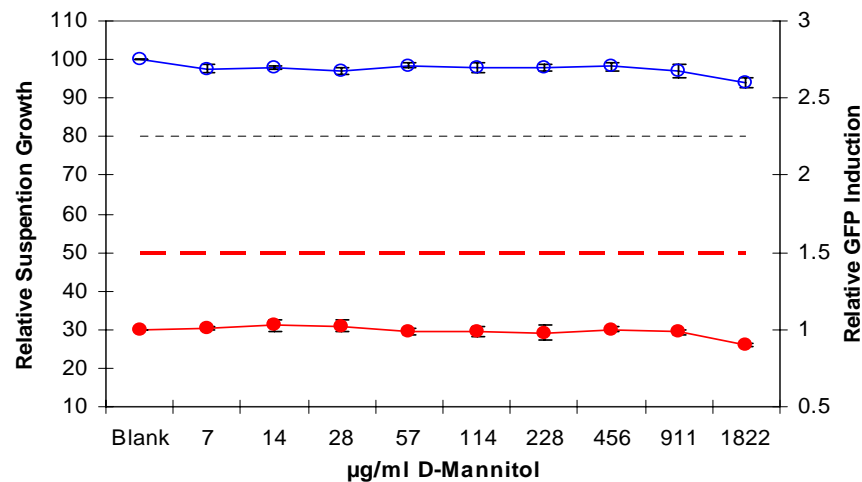
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Cellular biosensor systems for pre-clinical drug discovery

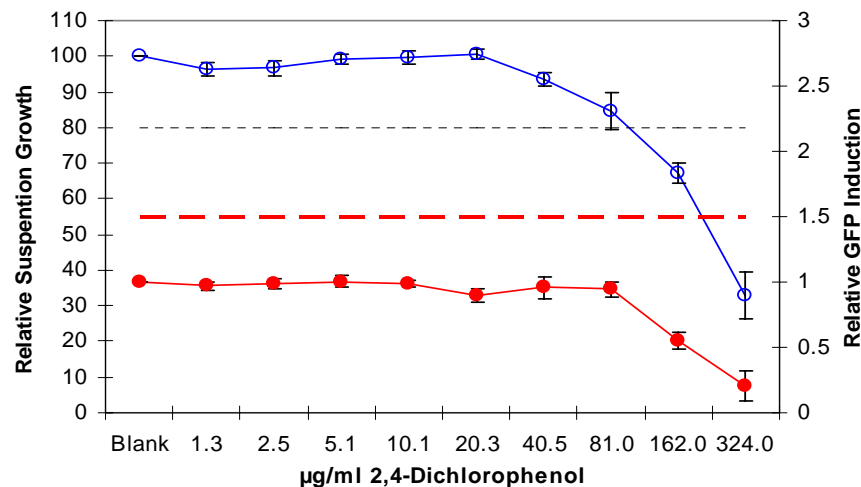
Non-genotoxins give negative results

GADD45a-EGFP reporter induction was not observed following treatment with any of the 33 non-genotoxic agents. (high specificity)

15 agents were not cytotoxic and did not induce the reporter at 10mM.



18 compounds tested to cytotoxic levels didn't induce the reporter. The 11 cytotoxic MNT 'unique' positives were all negative, including 2,4 DCP below.



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Agreement with Genotoxicity Assays

	Agreement with HC result		
	Positive	Negative	
Ames	18/30 *1	38/40	
MLA	30/31	31/39 *2	
in vitro CA/MNT	31/31	27/38 *2	
in vivo CA/MNT	27/30	19/23	

***1 Ames misses some eukaryotic genotoxins**
(mainly aneugens and nucleotide DNA synthesis inhibitors)

***2 The *in vitro* mammalian tests include 'false' positives**

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Prediction of genotoxic carcinogenesis

	Concordance	Sensitivity	Specificity
Ames:	92%	71	100
MLA:	88%	100	78
<i>in vitro</i> CA/MNT:	79%	100	63
<i>in vivo</i> CA/MNT:	89%	95	93
GADD45a-EGFP	98%	95	100

High Sensitivity without loss of specificity

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Screening sensitivities

1. **LOD: There are practical limits to the top concentration for screening**

-DMSO is Toxic, but compounds are stored at 10mM

-Screening is limited to 1% DMSO = 100µm

-DMSO is a weak reducing agent: (some compounds are water+, DMSO-)

**3 'weak' genotoxins would have been missed at this screening concentration:
EMS (156 µm), ENU (250 µm), Didanosine (2362 µm; equivocal)**

2. **Solubility**

There are no particular properties of the assay to make this different to other assays.

Detection: precipitation causes absorbance data to rise with concentration.

3. **Fluorescence**

Software gives alert from pure compound

Control data subtraction, nets off compound contribution

Fluorescence polarisation substantially improves data for strongly fluorescent compounds

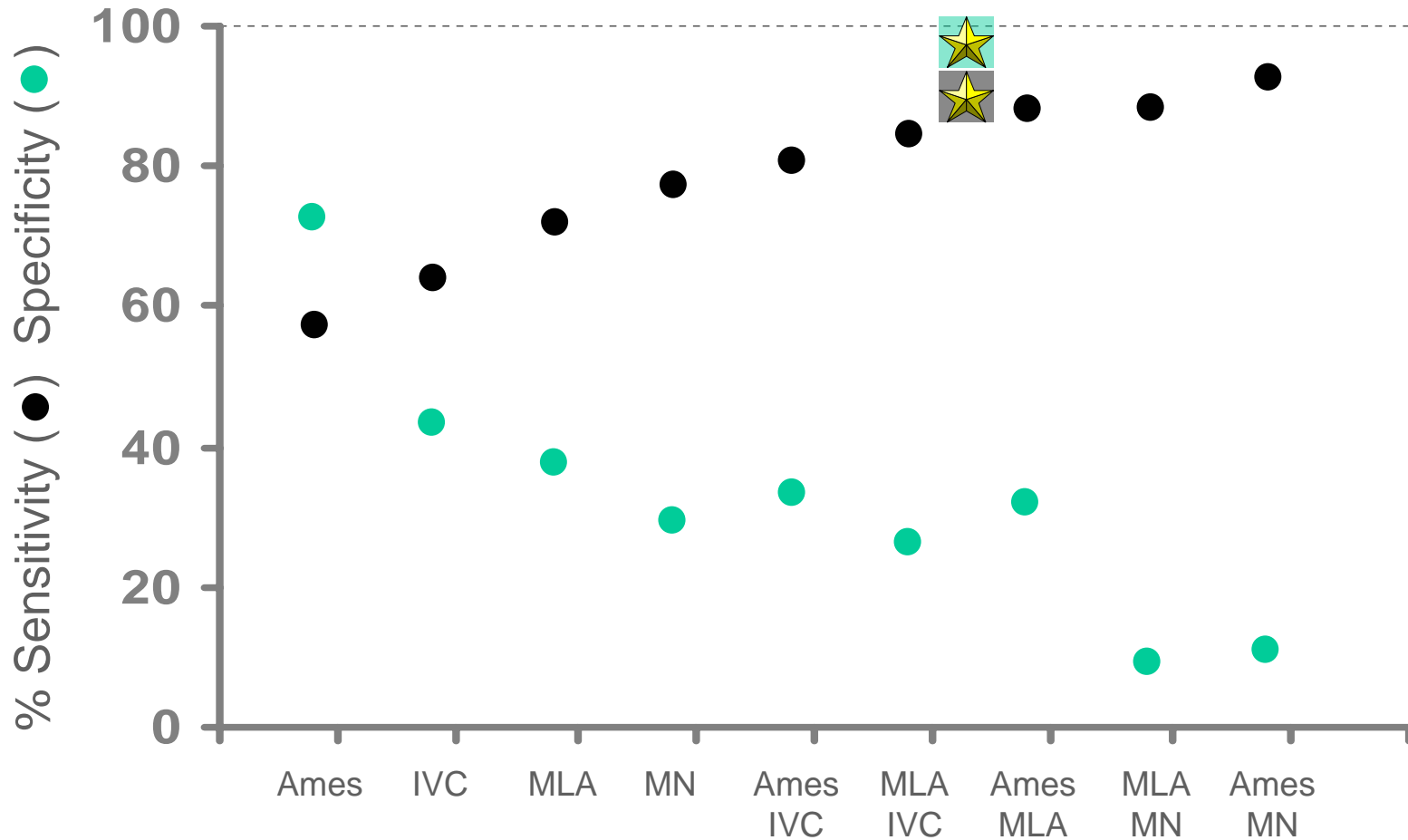
4. **Colour**

Interference if OD600 exceeds 1. Rare for pharma, more common in household goods and, of course, colorants

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Performance of the *in vitro* assays?

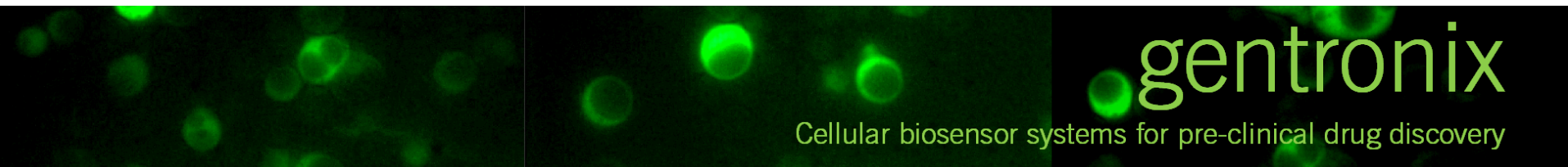


New data: marketed pharmaceuticals

MS in preparation

- 62 marketed pharmaceuticals tested using the same protocol as the initial validation exercise.
- Data set contains a large number of non genotoxins and non-genotoxic carcinogens.

no compounds uniquely positive with GADD45a



New Study: non-toxic, unique positives

6/8 negative with GADD45

	GADD45a result	
	24h	48h
1,2-Dichlorobenzene (MLA)	-	-
Aldicarb (MLA)	-	-
Diphenhydramine HCl(CA)	-	-
a-Methyl dopa sesquihydrate (MLA)	-	-
Sulfisoxazole (MLA)	-	-
Hydrochlorothiazide (MLA)	-	-
Hexachlorocyclopentadiene (CA)	+	-
Sodium diethylcarbamate trihydrate (MLA)	-/+	-/+

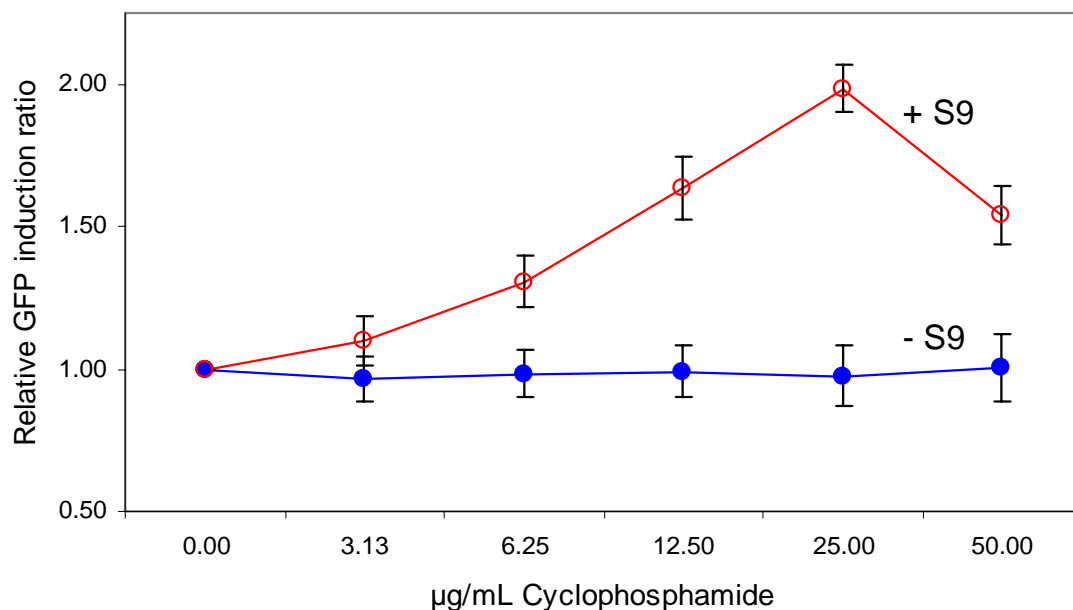
The GADD45a result can support weight of evidence proposals in safety assessment

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Published use of S9 (1)

- **S9 is fluorescent and light absorbing: not compatible with GFP**
- **Proof of principle with alternative lower throughput protocol (24 well plate, mix, wait 3h, wash, data collection after 24 hours)**



No single S9 protocol detects all promutagens!

Variables include:

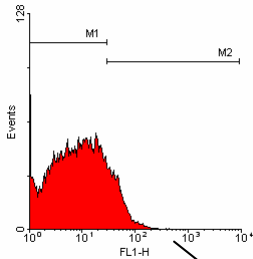
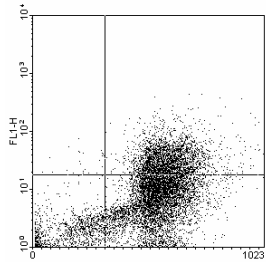
Rat, mouse, hamster, human, arochlor or penobarbitone induction, cofactor mixes etc etc ...

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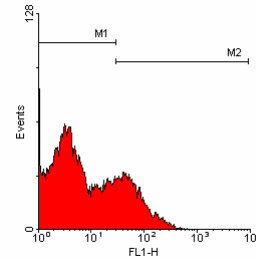
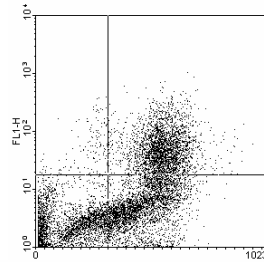
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New Flow cytometry studies...

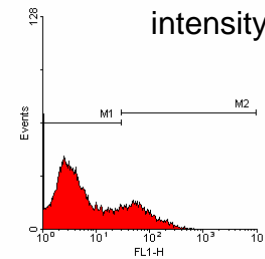
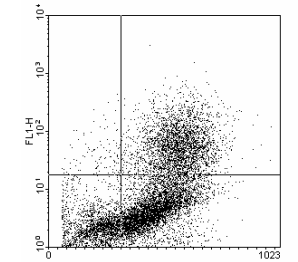
Non-induced



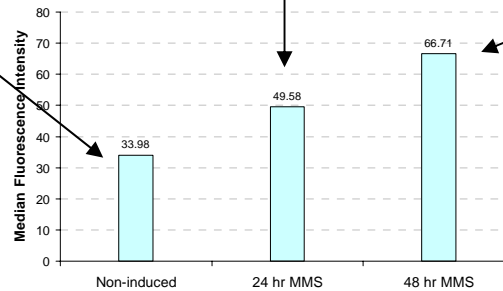
24hr
25ug/ml MMS



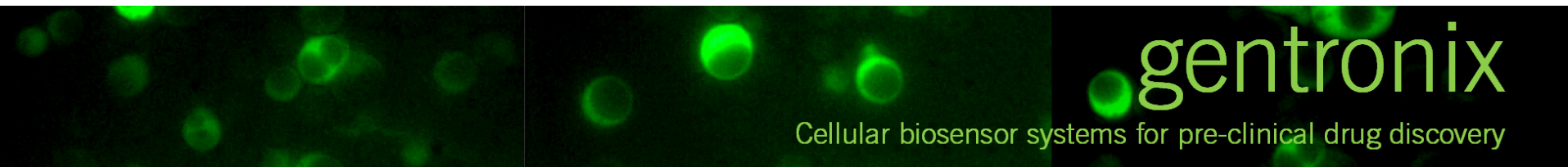
48hr
25ug/ml MMS



GFP fluorescence
intensity



**53 compounds
retested, no change
in genotoxicity call**



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New: S9 flow cytometry examples

3h S9 treatment, wash, 20h recovery, analysis by flow cytometry

B[a]P

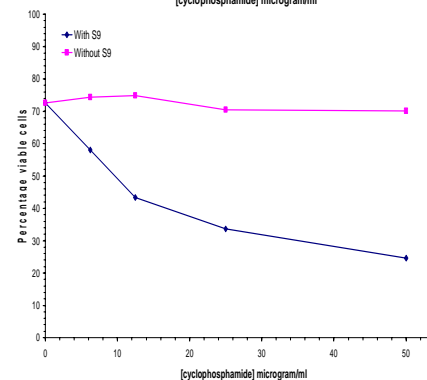
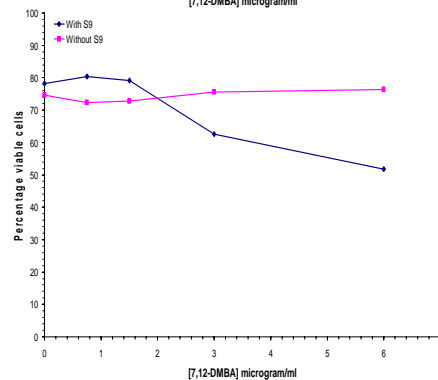
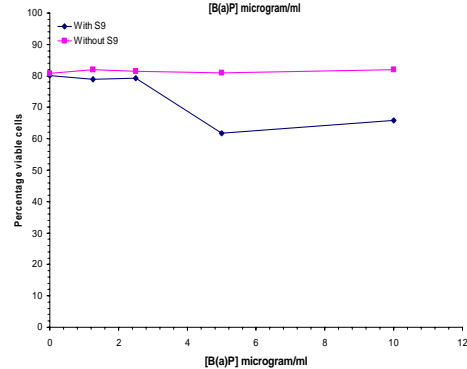
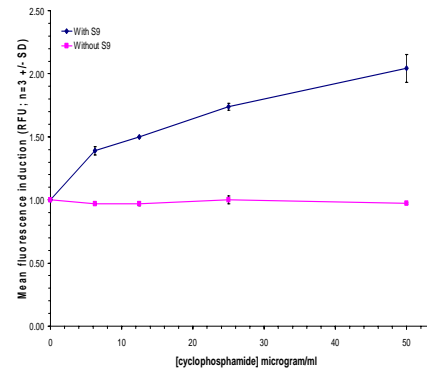
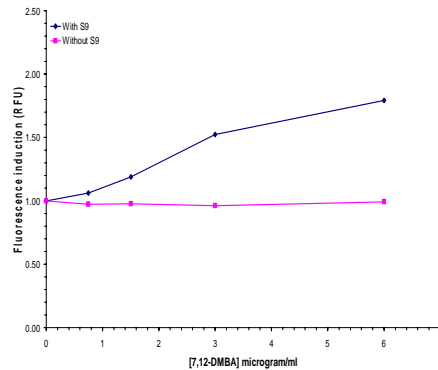
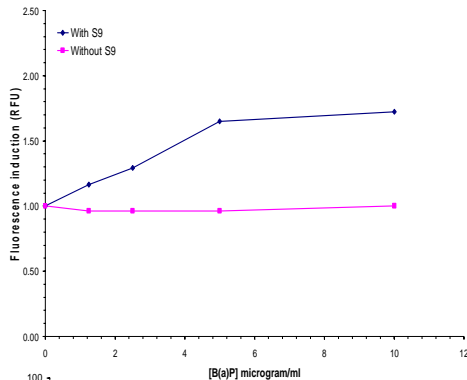
7,12 DMBA

Cyclophosphamide

Genotoxicity
(GFP induction)

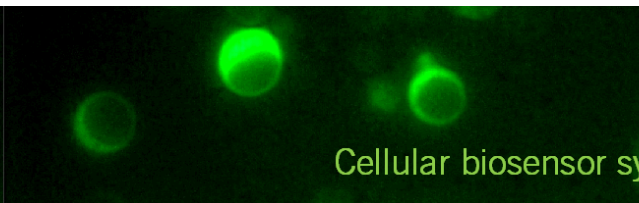
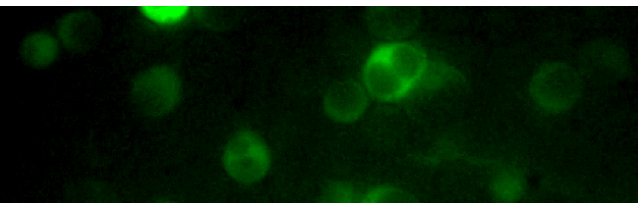
Cytotoxicity
(PI exclusion)

Also positive:
Aflatoxin B1,
6-aminochrysene,
2 amino anthracene



+S9

-S9



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Cellular biosensor systems for pre-clinical drug discovery

Robustness and reproducibility

- 4 laboratories: 2 pharma, 1 consumer products, UoM/Gentronix)
- 16 compounds, repeated 4 times
 - prepared as one batch, coded, frozen and distributed
- Media and Cell lines prepared as single batches for distribution;
- Training of multiple operators
- Questions – from ECVAM guidelines
 - and the thresholds robust? Yes
 - are the protocols sufficiently detailed? Yes, now!
 - within laboratory reliability? Yes, published
 - transferability? between laboratory variability Protocols refined

experienced microplate users get excellent reproducible results

practice makes perfect

inspect positive data – spikes etc

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Cellular biosensor systems for pre-clinical drug discovery

Early adopter programme

7 companies now have the assay running

A contract research company

- reproduced data from 11 positives and 9 negatives
- now providing testing service

International Pharma 1

- now tested 338 compounds
- positive hit rate higher than Ames

International Pharma 2

- now using in routine candidate selection

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Cellular biosensor systems for pre-clinical drug discovery

Applications

- **Screening**

- early hazard alerts for medicinal chemists
- improved quality of compounds for development (fewer genotox +ve)
- avoid costly late failure or mechanistic studies: phase1 delay, patents?

- **When to use it? Early!**

- high throughput Hit to Lead screening 100-1000s
- medium throughput Lead Optimisation 20-100 per project
- pre-regulatory Lead Candidate screening 5 per project

**When you have high specificity tools:
when positive means positive!**



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Cellular biosensor systems for pre-clinical drug discovery

Ranking unique positives

- 42 typically challenging compounds now tested
Ames negative, but... *in vitro* mammalian positive.
- Screening with the GADD45 assay helps segregate them

27 of the 42 were negative in the *in vivo* MNT – SAFE DRUGS?
26 of these were also negative with GreenScreen HC.
Exception: Ciclopirox Olamine, clastogen, unavailable *in vivo*

15 of the 42 were positive in the *in vivo* MNT
12 were also positive in GreenScreen HC
Exceptions
2,4-Dichlorophenol: (non carc);
Pyrazinamide: weak response *in vivo* (carc?);
Stavudine: nucleoside analogue, high conc *in vivo* pos

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GADD45a assay in summary

p53-dependent, genotoxin specific

detects all classes of genotoxin

good prediction of rodent genotoxic carcinogens

high sensitivity without loss of specificity

use for early hazard profiling, or late trouble shooting

needs no 'cut down' versions – HTS ready

simple, fast, consumes <1mg



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Cellular biosensor systems for pre-clinical drug discovery

Acknowledgments

NC3R^S

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and Reduction of Animals in Research

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- Jim Harvey
- Robert Rees
- Andrew Knight
- Paul Cahill
- Chris Jagger
- Louise Birrell



MANCHESTER
1824

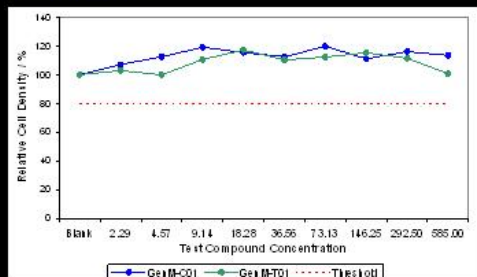
The University of Manchester

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Compound L	895.00	µg/ml
Test2-L	14.45	
00.01/1900	1	15.20
S Maskell	24.6	
% DMSO / Water	75	2.00
	150	
	75	

CYTOTOXICITY RESULTS



CYTOTOXICITY RESULT

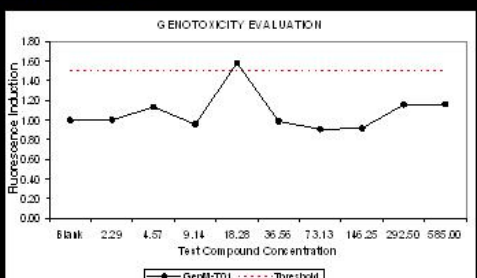
NEGATIVE

LEC: XXXXXXXXXX

Notab coding: XXXXXXXXXX

73.5 91.5 **PASS**

GENOTOXICITY RESULTS



GENOTOXICITY RESULT

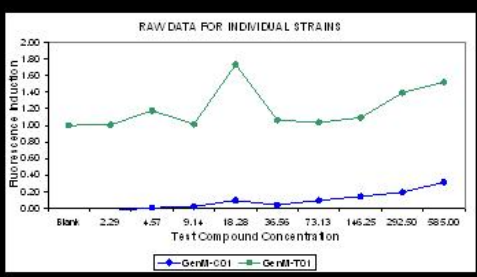
POSITIVE

LEC: XXXXXXXXXX

Notab-fluorescent: XXXXXXXXXX

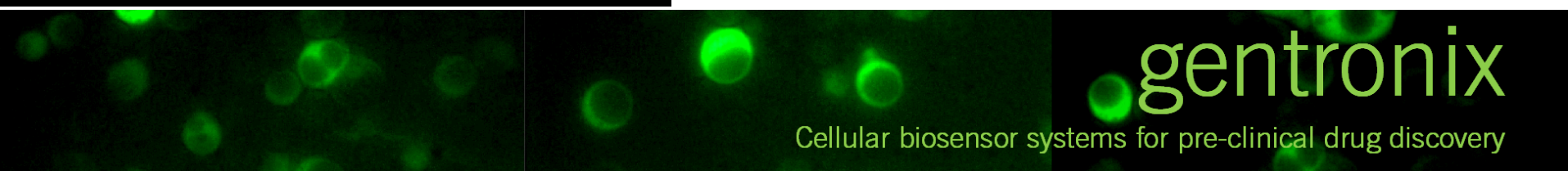
2.63 1.25 **PASS**

NOTES:



Visual inspection is important where positive data are obtained.

Data with spikes are readily identified.



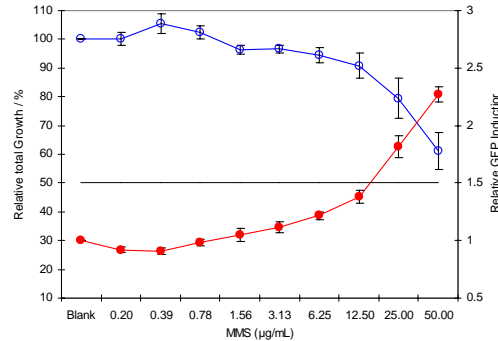
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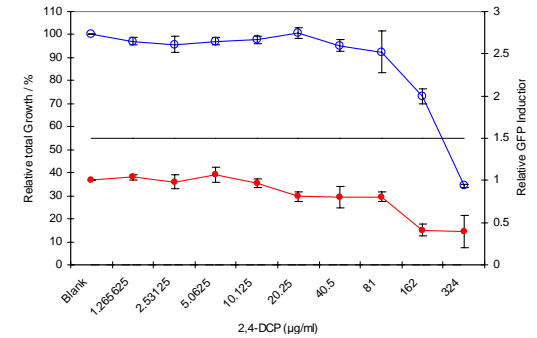
The distant future: HCS?

GreenScreen HC

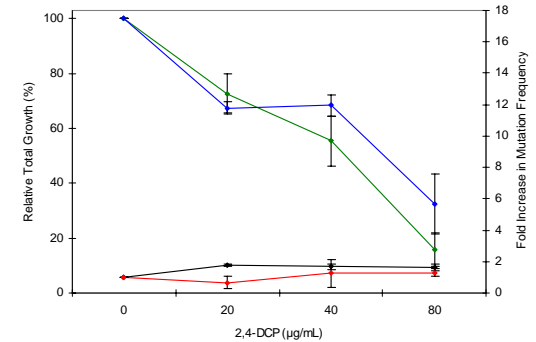
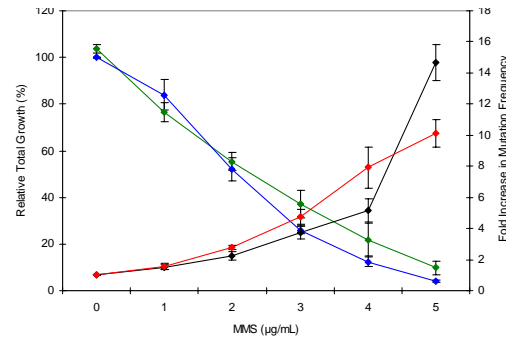
Genotoxin



Non-genotoxin



TK mutation



Micronucleus data

