

Assessment of the RAD54 genotoxicity assay: beyond the laboratory with GreenScreen EM

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Summary

Mutagenesis recently published the results of a screening validation exercise for the RAD54 genotoxicity and cytotoxicity assay, available commercially as GreenScreen (Vol 19: 105-119, 2004). In the assay, genotoxicity is assessed from GFP accumulation, driven by the DNA damage inducible RAD54 gene promoter. Cytotoxicity is measured by relative total growth.

The rigorous methodologies applied in pharmaceutical safety assessment are often either impractical to apply outside the laboratories of pharmaceutical companies and universities. We present results of some studies using this assay in cases where we have departed from the published microplate protocol.

- In a project sponsored by the DTI BioWISE programme, environmentally derived samples were assessed using a portable instrument alongside regulatory *Daphnia* toxicity tests. The genotoxicity aspect of the assay functioned well in a range of sample types (cloudy, coloured etc). Cytotoxicity data correlated well with the *Daphnia* data.
- A project sponsored by the EC in which an effluent treatment process was assessed, again using the portable instrument. Differences in toxicity profiles from samples taken at various times during sampling show how simple tests can give an indication of process effectiveness.

RAD54 Reporter Assay Protocol

The assay method is illustrated in Figure 1. 1 ml of each sample/dilution was combined with 1 ml of the yeast in growth media in a disposable acrylic cuvette, sealed with an adhesive breathable membrane and left overnight to incubate, ideally at 25–30°C. At least one non-toxic control was also prepared in which pure water was used as the test sample. Before measurement, the contents of the cuvette were thoroughly mixed by shaking or repeated pipette aspiration to re-suspend the cells. Fluorescence (F) and absorbance (A) data was collected using the YETI, shown in Figure 2 and processed to give a measure of cytotoxicity (A: reduction in cell proliferation and thus final cell density; Figure 3) and genotoxicity (F/A: increase in brightness showing induction of RAD54 gene; Figure 4).

For samples, which contained particulate matter, comparative cuvettes were prepared containing the corresponding dilution of the sample and growth media alone, without cells. Optical density values determined from these cuvettes were subtracted from the respective assay cuvettes to correct for sample turbidity. This correction worked well since insoluble particulate matter commonly found in environmental samples is largely unaffected by the presence of the yeast cell culture. A basic qualitative (highly toxic, toxic or non-toxic) result can be obtained from a single cuvette measurement by comparison to a blank, non-toxic control. However to obtain a quantitative result an EC50 (concentration for 50% effect) was determined from the results of assays on a linear range of typically 8 to 10 concentrations. Thresholds: fluorescence induction >1.3 = positive genotoxic response; reduction in cell number >20% = positive cytotoxic response.

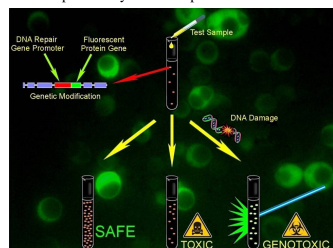


Fig. 1 Schematic diagram of the GreenScreen Assay.



Fig. 2 YETI – Yeast Environmental Toxicity Indicator

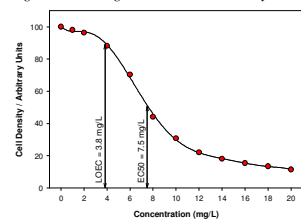


Fig. 3 Cytotoxicity profile for 3,5-dichlorophenol

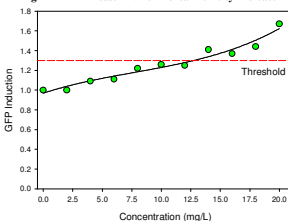


Fig. 4 Genotoxicity profile for nickel(II) ions

1. Assessment of pure compounds and effluent samples using GreenScreen EM

A range of environmentally relevant substances has been evaluated using the assay, including solutions of metal ions, solvents and pesticides. Preliminary data comparing the yeast assay's response to that of a standard *Daphnia* test in the analysis of the toxicity of 34 varied industrial waste effluents are presented. The sensitivity to a wide range of substances (Table 1) and effluents (Table 2) suggests the assay should be useful for environmental toxicity monitoring. Data reproduced from *Journal of Environmental Monitoring* 6: 71-79 (2004).

Table 1 Summary of cytotoxicity and genotoxicity results for the pure compounds tested

Class	Compound	Cytotoxicity EC50 (mg L ⁻¹)	LOEC (mg L ⁻¹)	Genotoxicity Evaluation ^a	LOEC (mg L ⁻¹)	Solvent	
Metal Ions	Cadmium(II)	0.032	0.019	N	—	Water	
	Copper(II)	0.052	0.023	N	—	Water	
	Chromium (VI)/Dichromate	0.55	0.041	N	—	Water	
	Mercury(II)	0.47	0.18	N	—	Water	
	Nickel(II)	21	1.7	P	14	Water	
	Zinc(II)	126	4.7	N	—	Water	
	Lead(II)	—	30	N	—	Water	
	Solvents	Methanol	24000	3700	N	—	Water
		Ethanol	25000	4200	P	24000	Water
		Dimethyl sulfoxide	41000	9200	P	22000	Water
Cycloheximide		0.015	0.0023	N	—	Water	
Pesticides	2,4-D/2,4-Dichlorophenoxyacetic acid	55	7.8	N	—	Water + 1% DMSO	
	Paraquat/Methyl viologen	101	11	P	30	Water	
	Nitrogen mustard/Mechlorethamine HCl	0.32	0.047	P	0.2	Water	
	Sodium hypochlorite	2.6 ^b	1.5 ^b	—	—	Water	
	3,5-Dichlorophenol	7.5	3.8	N	—	Water	
Others	Sodium dodecyl sulfate (SDS)	33	3.3	N	—	Water	

^a Genotoxicity evaluation, N = negative and P = positive. ^b ppm available chlorine

The sensitivity of the assay has proved to be broadly similar to other cell based assays in the determination of pure compounds, and demonstrates significant correlation with the standard *Daphnia* screen in the analysis of 34 whole effluent samples, and thus the assay is proposed as applicable to the determination of toxicity in contaminated effluent and surface water samples. In this exercise GreenScreen demonstrated equivalent or higher sensitivity for cytotoxicity compared to the standard *Daphnia* screen in 26 out of 34 cases. 9 effluents tested positive for genotoxicity. The assay in its current form is not sensitive enough to determine trace contaminants in drinking waters, especially in the case of organic compounds. Genetically modifying the yeast cells to produce strains with more permeable cell walls or disabled membrane transport systems improves the sensitivity for this particular application (Manuscript in preparation).

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2. Wastewater Treatment Study

In a recent study, GreenScreen EM was used to monitor the effect of ozone and electrochemical oxidation treatment methods on the toxicity of coloured dye effluents. Colour and foam are undesirable properties in an effluent. The former is readily reduced by oxidative reactions including treatment with ozone or electrochemical treatment. Figure 5 below shows the successful decolourisation of a sample of dye effluent.

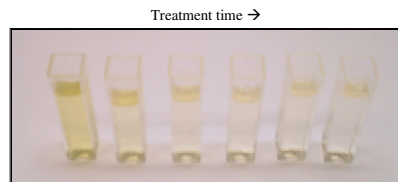
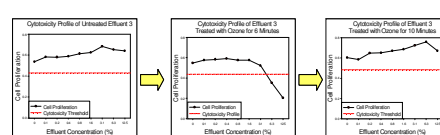
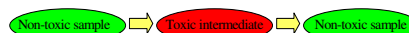


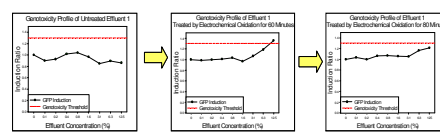
Fig. 5 Decolourisation of a dye effluent with treatment.

Such treatment might be expected to create reactive intermediates in much the same way as biological oxidation processes by the mixed function oxidases (CYP 450s), with a corresponding toxic potential. Samples of effluents were taken at various times during treatment and assessed both for genotoxicity and cytotoxicity.



Example 1.

The untreated sample slightly promoted growth, and thus may have contained available sources of carbon or nitrogen. The mid-treatment sample showed pronounced toxicity which was subsequently removed after full treatment



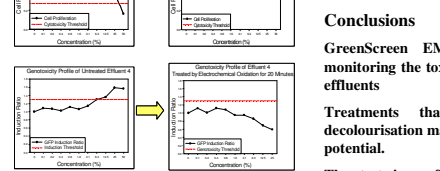
This sample was not genotoxic in the raw form but the mid-treatment sample was genotoxic. Full treatment reduced the genotoxic response



Example 2.

The untreated sample was toxic at high concentrations but following treatment became a substrate for growth.

The untreated sample was genotoxic at high concentrations but was inactivated by the oxidative treatment.



Conclusions

GreenScreen EM is an effective method for monitoring the toxic and genotoxic properties of dye effluents

Treatments that appear successful through decolourisation may not be effective at reducing toxic potential.

The test is useful in developing and optimising treatment processes, and for long term monitoring of the performance of such processes.

Table 2 Summary of cytotoxicity and genotoxicity data for a range of industrial effluents comparing the yeast assay with a standard *Daphnia* test

Sample ID	Description ^a	pH	Daphnia Screen			YEAST			Genotoxicity	
			Toxicity range (%)	EC50 (%)	EC10 (%)	EC50 (%)	LOEC (%)	Comparison of EC50 response ^{b,c}	Result	LOEC (%)
02-0225	Transparent	11.5	1.0-3.2	1.5	4.8	1.1	<<	<<	N	—
02-0226	Br/Transparent/Particulate	8.5-9.0	1.0-3.2	1.3	3.3	0.72	>10	>50	N	—
02-0227	Br/Transparent	7.5-8.0	>10	>10	>10	>10	>10	>10	(NT)	N
02-0228	Br/Transparent	8	>10	>10	>10	>10	>10	>10	(NT)	P
02-0229	Br/Opaque/Particulate	9	0.32-1.0	0.34	11	1.5	<<	<<	N	—
02-0252	Pale Y/Transparent	8	>10	>10	>10	>10	>10	>10	(NT)	P
02-0253	Br/Opaque/Particulate	7	>10	>10	>10	>10	>10	>10	(NT)	P
02-0264	Bl-Bk/Opaque/Particulate	2	0.32-1.0	0.69	3.8	0.2	<<	<<	N	—
02-0364	Bk-Pu/Transparent/Particulate	2	3.2-10	5.7	3	0.17	>>	>>	N	—
02-0365	Br/Transparent/Particulate	9.5	<<1.0	<1.1	2.5	0.15	<<	<<	N	—
02-0366	Br-Pu/Transparent/Particulate	6.5	>10	>10	6.3	0.94	>>	>>	N	—
03-0078	Pale Y/Opaque/Particulate	7	1.0-3.2	1.8	>50	>50	>>	>>	P	5
03-0079	Br/Opaque/Particulate	7	<<1.0	<1.1	8.9	0.44	<<	<<	N	—
03-0080	Gr/Opaque/Bk/Particulate	7	3.2-10	4.0	23	6.2	>>	>>	N	—
03-0081	Y-Br/Opaque/Particulate/Volatile	12	0.32-1.0	0.46	1.1	0.3	>>	>>	N	—
03-0082	Bk/Opaque/Particulate	6	>10	>10	13	4.3	>>	>>	(Dap > 10)	P
03-0146	Bl/Clear	5	0.32-1.0	0.57	0.43	0.28	>>	>>	P	0.05
03-0147	Pale Y/Clear	7.0-7.5	>10	>10	>10	>10	>10	>10	(NT)	P
03-0148	Pale Y/Opaque	7	>10	>10	>10	>10	>10	>10	(NT)	N
03-0149	Br-Rd/Transparent	10.5-11	<<1.0	<1.1	8.8	0.9	<<	<<	N	—
03-0150	Transparent/Particulate	6.5	>10	>10	>10	>10	>10	>10	(NT)	N
03-0175	Transparent/Particulate	7	>10	>10	>10	>10	>10	>10	(NT)	N
03-0176	Pale Br/Transparent	9	5.0-5.5	3.2-10	4.4	0.86	0.43	>>	N	—
03-0177	Transparent	9	>10	>10	>10	>10	>10	>10	(NT)	N
03-0179	Y-Br/Transparent/Particulate	7.5	>10	>10	>10	>10	>10	>10	(NT)	P
03-0204	Rd-Br/Transparent/Volatile	7	>10	>10	11	1.5	>>	>>	(Dap > 10)	N
03-0212	Rd-Pu/Transparent/Volatile	2	3.2-10	4.6	6.0	1.1	>>	>>	N	—
03-0213	Y-Br/Transparent/Particulate	9.5	>10	>10	19	12	>>	>>	(Dap > 10)	N
03-0214	Opaque/Particulate	7	>10	>10	44	14	>>	>>	(Dap > 10)	N
03-0215	Y-Br/Transparent/Br/Particulate	9	>10	>10	47	26	>>	>>	(Dap > 10)	N
03-0216	Pk-Br/Opaque/Particulate	9	>10	>10	1.3	0.15	>>	>>	N	—
03-0252	Gy-Gr/Transparent/Particulate	9	>10	>10	27	11	>>	>>	(Dap > 10)	N
03-0253	Y-Gr/Transparent/Bk/Particulate	9	>10	>10	20	14	>>	>>	(NT)	P
03-0338	Rd-Br/Opaque/Volatile	3	>10	>10	4.3	0.57	>>	>>	N	—

^a Effluent Colours: BK, Black; Bl, Blue; Br, Brown; Gr, Green; Gy, Grey; Pk, Pink; Pu, Purple; Rd, Red; Y, Yellow. ^b Equivalent results: (NT), Non-toxic in both *Daphnia* and Yeast concentration ranges tested; = (Dap > 10), Toxicity detected in Yeast above the concentration range tested in *Daphnia* screen; = Yeast EC50 in the toxic concentration range of *Daphnia* screen. ^c Differing Results: <<, Yeast test is less sensitive for toxicity compared to *Daphnia* screen; >>, Yeast test is more sensitive for toxicity compared to the *Daphnia* screen.