

# Flow cytometry increases the spectrum of compounds detected by the *GADD45a-GFP* genotoxicity assay; assessment of metabolites and compounds with interfering properties



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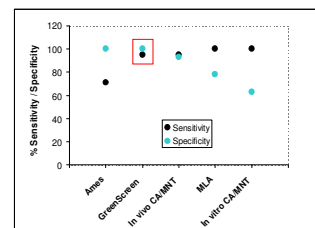


## Introduction

Hastwell *et al.*, (2006) described the validation of the *GADD45a-GFP* (GreenScreen HC™) *in vitro* genotoxicity assay which uses TK6 lymphoblastoid cells. This validation (of 75 compounds) revealed the assay to have high sensitivity to genotoxic carcinogens without compromising specificity. Recent reports have highlighted a significant problem with the specificity (ability to correctly predict non-carcinogens as negative) of the current mammalian *in vitro* regulatory genotoxicity tests. For the validation compounds, GreenScreen HC showed levels of sensitivity comparable to the other *in vitro* mammalian assays whilst possessing a much higher level of specificity than these assays (see Figure 1).

ICH guidelines for *in vitro* genotoxicity assessments require testing to be carried out with and without a source of metabolic activation. Commonly this is provided by incubation with 'S9' liver extracts prepared from rats, exposed to Aroclor-1254. S9 however has certain physical properties which interfere with fluorescence data collection in a microplate reader. Additionally, a further challenge in microplate data collection are compounds which can be optically interfering (e.g. have light absorbing and/or auto-fluorescent properties).

Here we demonstrate the development of a flow cytometric method (FCMM) which reproduces microplate reader data. We also show that the assay is able to detect pro-genotoxins and compounds with light interfering properties. Finally we demonstrate the transferability of this FCMM to a collaborator's site in Italy with initial validation data for 4 compounds



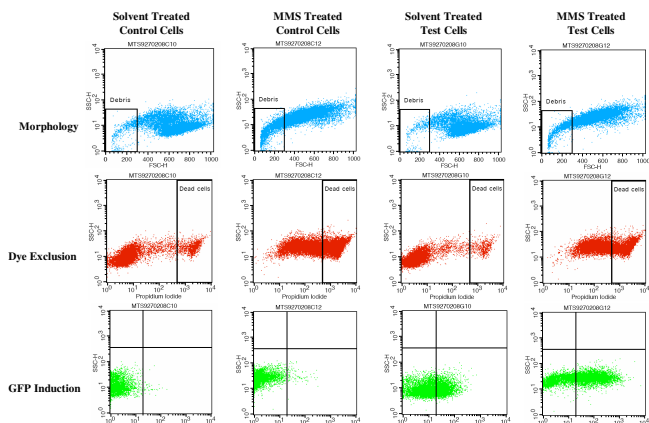
**Figure 1.** Percentage sensitivity and specificity for rodent genotoxic carcinogenicity from a 75 compound dataset. Data points are shown for the ICH battery tests and GreenScreen HC.

## Methods

The 96-well microplate assay allows genotoxicity assessment for 4 compounds over 9 dilutions and gives results within 48 hours. All compounds were tested according to ICH guidelines (5 mg/ml, 10 mM, limit of solubility/cytotoxicity). For assessment of pro-genotoxins, cells were exposed to compound and 1% S9 mix for 3h. They were then washed and allowed to recover in fresh medium for 45 hours. Flow cytometric analysis was carried out using either a BD FACScalibur™ or FACScan™. Background fluorescence was removed and mean GFP channel intensities normalised to give fold induction values over negative control. Propidium iodide exclusion was used to determine cell survival.

## GreenScreen HC Data Collection by Flow Cytometry

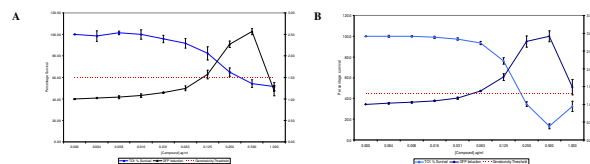
The standard GreenScreen HC assay uses a microplate reader for the collection of fluorescence and absorbance data. Using a flow cytometer, we have now developed a gating strategy (Figure 2) which allows the collection of data and the generation of comparable results for genotoxicity.



**Figure 2.** FCMM gating strategy restricts collection of GFP fluorescence to events of cellular morphology and capable of containing GFP. An increase in fluorescence of the GFP reporter cell population can clearly be seen when cells are exposed to the potent genotoxin, MMS. Measurements for viability include all events except those within the debris region and genotoxicity calculations are based on events that lie outside the debris and dead cell regions.

## FCMM reproduces data collected in a microplate reader

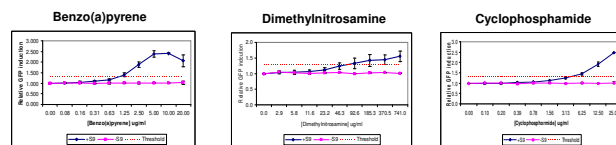
The FCMM accurately reproduces the data collected using a microplate spectrophotometer (see Figure 3). Having successfully developed and evaluated the FCMM we attempted to assess whether this method would allow the collection of genotoxicity data for compounds known to require biotransformation. Data presented here show that the method allows the collection of this data and publications are in preparation detailing method development and validation.



**Figure 3.** Example showing data collection using a microplate reader (A) is reproducibly generated using FCMM (B) for 4-nitroquinoline-N-oxide.

## Detection of pro-genotoxins

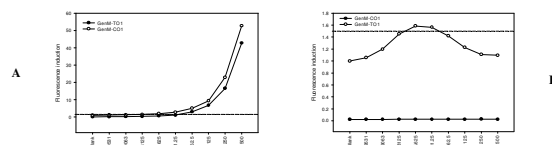
The presence of the S9 causes difficulties in the interpretation of GreenScreen HC data collected using a microplate reader. Figure 4 shows an example of how FCMM allows the assessment of pro-genotoxins.



**Figure 4.** Genotoxicity data for 3 known pro-genotoxic compounds. Data were collected using the described FCMM method following 48hrs incubation and positive results for genotoxicity were seen.

## Detection of optically interfering compounds

The implementation of the developed FCMM has also allowed compounds with optically interfering properties to be assessed for genotoxicity. Figure 5 gives an example of such a compound, 4-nitro-o-phenyldiamine.



**Figure 5.** Data collection by FCMM (B) reduces the effects of compound auto-fluorescence compared to microplate reader collection (A). FCMM removes compound auto-fluorescence allowing GFP signal and genotoxicity to be detected.

## Transfer of the Protocol

To assess the reproducibility of the FCMM it was transferred to a laboratory in Angelini Research Center, Italy and a standard *GADD45a-GFP* quality control plate was run. Expected positive results were clearly seen for 4-NQO and MMS. See Table 2 for results summary.

Compound	Top Test concentration (ug/ml)	Angelini Genotoxicity result	LEC (ug/ml)	In house Genotoxicity result	LEC (ug/ml)
4-NQO	1.0	Positive	0.063	Positive	0.125
MMS	50.0	Positive	3.13	Positive	3.13
2,4-DCP	324.0	Negative	-	Negative	-
1% DMSO	1% DMSO	Negative	-	Negative	-

**Table 1.** Result for genotoxicity of 4 compounds tested at Angelini using the FCMM described above.

1. Hastwell *et al.*, (2006) *Mutation Research* 607, 160-175. High-specificity and high-sensitivity genotoxicity assessment in a human cell line: validation of the GreenScreen HC genotoxicity assay

## Conclusions

1. The development of the described FCMM permits the collection of GreenScreen HC data using flow cytometry with reproducible results compared to those collected using a microplate spectrophotometer.
2. The use of FCMM allows detection of pro-genotoxins and compounds with optically interfering properties, increasing the spectrum of compounds which can be detected in the assay.
3. We have shown that the FCMM is transferable: data generated at Angelini Research Center show good reproducibility with data generated at Gentronix.