

43: FLOW CYTOMETRIC ASSESSMENT OF PROMUTAGENS IN THE GADD45A-GFP GENOTOXICITY SCREENING ASSAY

Chris Jagger, Matt Tate, Paul Cahill, Nick Billinton and **Richard Walmsley**
Gentronix Ltd and The University of Manchester, UK

Introduction

The IWGT Strategy Expert Group emphasized the importance of metabolic conversions in risk assessment, and drew attention to deficiencies in current methods [Ku *et al.*, *Mutat. Res.* 627 (2007) 59-77]. We recently described the GADD45a-GFP *in vitro* genotoxicity reporter assay in TK6 lymphoblast cells [Hastwell *et al.*, *Mutat. Res.* 607 (2006) 60-175]. An initial validation study (75 compounds) revealed high specificity to genotoxic carcinogens without compromising sensitivity. The study excluded promutagens because S9 post-mitochondrial liver extracts confound spectrophotometric data assessment.

Here we show that flow cytometry allows collection of data from samples exposed to S9. We present genotoxicity data from a collection of promutagens tested using an S9 protocol and discuss limitations in metabolite screening.

These preliminary data demonstrate that flow cytometry provides an effective genotoxicity assessment of metabolites generated by S9 in the GADD45a-GFP assay. The method succeeds partly because flow cytometric measurements are inherently less affected by the colour and fluorescence of extracellular fluids, and partly because one can exclude non cell-like particles (membrane fractions and cell fragments) from the analysis. This might be a general method for assessment of metabolites generated by other mammalian S9 preparations in this assay.

Flow cytometric reporter assessment detects promutagens after S9 exposure

Fig 1. S9 increases the GFP fluorescence of reporter cells in the presence of 25 µg/ml Cyclophosphamide (CPA)

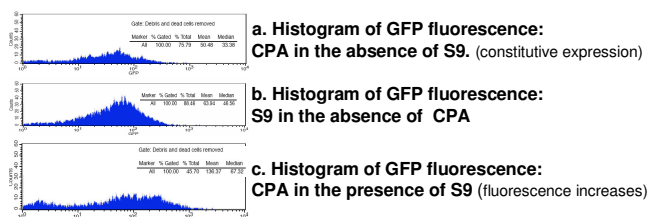


Fig 2. S9 increases the proportion of propidium iodide positive cells in the presence of 25 µg/ml CPA

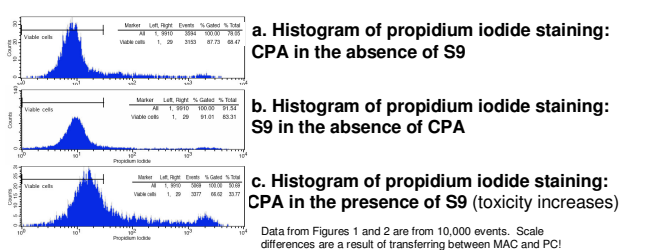


Fig 3. Integration of results from flow analysis of cells. Increasing concentration of CPA (+S9) reveals dose dependent increase in GFP fluorescence and decreasing viability

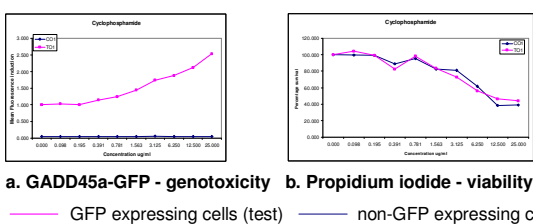


Fig 4. Flow reproduces standard (-S9) spectrophotometric data

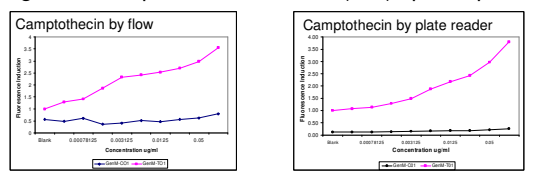
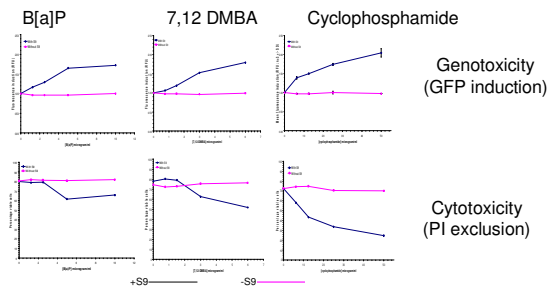


Fig 5. Dose-dependent induction of GFP by promutagens in the presence of S9



So far, negative, non-promutagens are all negative with S9

Method

Cells were incubated for 3h with test compound and 1% S9 mix (from MolTox, Aroclor 1254-induced male Sprague-Dawley rat). They were then washed and resuspended in fresh medium for 20 hours. Samples were analysed using a BD FACSCalibur™.

Preliminary Data:

Preliminary data for >20 promutagenic compounds has been obtained. However, the protocols for the use of S9 with GreenScreen HC have been further refined since this poster presentation. If you would like to receive an update of our latest complete data set please contact:

richard.walmsley@gentronix.co.uk

Discussion

It is clear from the literature that different promutagens require different protocols for their detection. Relevance of positive results to human exposure is not always proven. Why? The source species of the liver extract affects the spectrum of metabolic enzymes. S9 itself has the inherent disadvantages of phase 2 depletion.

Here we have only looked at a single protocol, but picked up a good spectrum of promutagens. Whilst initially surprised at the negative results with 2AAF, this compound is only positive in other *in vitro* mammalian tests at high concentration – with and without S9.

Are S9 results reliable?

Kirkland *et al.* (2006) and Matthews *et al.* (2006) warned us of the inaccuracy of the current *in vitro* mammalian tests: should we assume the same levels of inaccuracy in the presence of S9? Might there be more unique positives when predominantly phase 1 metabolism is applied? Will the GADD45a assay maintain specificity with S9? We are currently testing 'true negatives'.

Can you help validate the new S9 protocols?

We would like to know how accurately the S9-supplemented GADD45a-GFP assay performs. Have you got failed compounds that are only positive with S9 and were subsequently positive in *in vivo* tests or cancer assays, or happier stories where S9 positive compounds were subsequently negative in *in vivo* test or carcinogenicity assays? We would like to test them. No proprietary information will be shared without mutual agreement.

ps...using hepatocytes instead of S9

AbCellute (Cardiff) provided us with stabilised 1^{ary} rat liver hepatocytes, and we used them as a reagent in much the same way as we have used S9. Preliminary data from two promutagenic compounds were positive. From a theoretical standpoint these cells are preferred due to better representation of phase 2 metabolism.