

# Genotoxicity Screening Steps Up a Gear: Validation of a Higher Throughput Protocol for the GreenScreen HC GADD45a-GFP Genotoxicity Screening Assay

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## Introduction

The GreenScreen HC genotoxicity assay measures GADD45a (growth arrest and DNA damage repair) gene induction in the human lymphoblastoid TK6 cell line. Test cells incorporate a GFP reporter based on the proper regulation of the human *GADD45a* gene, which mediates the adaptive response to genotoxic stress. The patented GFP reporter also includes p53 regulatory elements.

**Several features of the assay make it amenable to high throughput screening:**

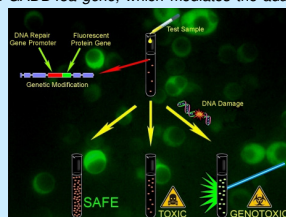
- Optical Endpoints** – Non-invasive measurement of induced GFP fluorescence indicates genotoxic potential of the test article, whilst measurement of optical absorbance quantifies cytotoxicity by reduction in proliferation.
- Suspension Cell Line** – Simplifies routine cell passage and handling.
- Robust Cells** – Enables the use of robotic liquid handling automation.
- Fast Growing** – Rapid turnaround with results in 24 / 48 hours.

### Initial Assay Validation:

Initial validation of the GreenScreen HC assay included an analysis of 75 well characterised genotoxic and non-genotoxic compounds with diverse mechanisms of action (*Hastwell et al. Mutat. Res. 2006*). This study along with an additional 70 pharmaceutical compounds has demonstrated that GreenScreen responds positively to all classes of genotoxic damage with high sensitivity for genotoxic carcinogens (> 80%) without compromising high specificity (> 90%), i.e. the discrimination of non-carcinogens. Compounds were tested in 96 well microplate format, with 4 compounds tested over 9 dilutions per microplate. Compounds were tested up to a concentration of 10 mM or the limit of solubility or cytotoxicity, often with a range finding experiment, followed by at least 4 replicate assays to corroborate results.

### This Study: High-Throughput Validation with the LOPAC Chemical Library of 1266 Compounds:

The aim of this study was to reformat the GreenScreen HC assay for higher throughput, more suited to automated testing of larger compound collections. The new format tested 12 compounds per 96 well microplate, each over 3 serial dilutions from a fixed concentration (100  $\mu$ M) and testing only once where possible. This has allowed quantification of the hit rates for GreenScreen HC when used in much wider, pharmaceutically relevant chemical space and tested that high specificity is maintained. The types of compound GreenScreen HC responds to have been characterised and the limitations of compound properties such as solubility and autofluorescence have been assessed. Finally the GreenScreen HC assay results have been compared to an *in silico* approach to genotoxicity assessment using the DEREK for Windows software.

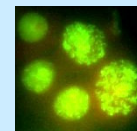


## LOPAC Chemical Collection

- 1266 Pharmacologically active compounds
- All tested from a fixed concentration of 100  $\mu$ M
- All major target classes represented (antiproliferatives, enzyme inhibitors, antibiotics, cell cycle regulators, apoptosis inducers, GPCR ligands...)
- Test consumes 10  $\mu$ l aliquots of 10 mM stock in 100% DMSO per compound

## Results Overview

- 7.3% of compounds (92) were **genotoxic**
- 33% of compounds (419) were **cytotoxic**
- 20% of **cytotoxic** compounds were **genotoxic**
- 89% of **genotoxic** compounds were **cytotoxic**



- Prevalence of genotoxicity similar to other proprietary collections
- 100  $\mu$ M captures the active toxicological concentration range
- Cytotoxicity alone does not cause genotoxicity response

## Physicochemical Properties

- 22% of compounds had significant autofluorescence but only 5 (0.4%) were too autofluorescent for analysis
- Only 5 compounds (0.4%) precipitated at test concentrations
- 115 compounds (11%) visibly coloured in solution

## GreenScreen HC positives are potent carcinogens

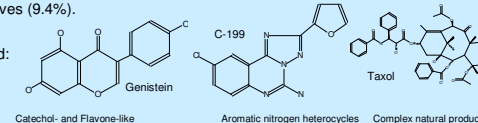
Carcinogenic Potency: 8 of the GreenScreen HC positives were in CPDB, **all were in the top 2% for potency in this collection**

47 non-carcinogens were readily identified and **only 1 GreenScreen HC positive was non-carcinogenic** - it was also positive in other *in vitro* assays: methotrexate. **High specificity prevents incorrect hazard classification.**

Over 50% of compounds listed in referenced carcinogenicity databases had positive data - cancer studies are more likely when a risk has been suggested by *in vitro* studies or conceived exposure. Only 14 were consistently positive for carcinogenicity between sexes and between species.

## Differences between *in silico* and *in vitro* prediction

- Only 97 LOPAC chemicals (7.7%) were found in PDR, NTP, CCRS, IARC, CPDB because many are development chemicals. The data do not discriminate genotoxic and non-genotoxic modes of carcinogenesis because it is not a trivial analysis. This limits our own analysis.
- 427 LOPAC compounds (34%) alerted for Chromosome Damage, Carcinogenicity and/or Mutagenicity in DEREK for Windows (v. 10) 92 LOPAC compounds (7.3%) were positive with GreenScreen HC **Why the big discrepancy?**
  - Over-representation of particular chemical classes? Probably yes (see Table below)
  - Not all chemicals within an alerting class are actually genotoxic/carcinogenic, merely plausible.
  - The specificity of the *in silico* alerts is lower than the *in vitro* alerts: 47 compounds were negative in all cancer studies. GreenScreen correctly predicted 98% of non-carcinogens: DEREK correctly predicted only 66% of non-carcinogens **High specificity prevents incorrect hazard classification** (DEREK mis-classified 34% as potentially hazardous)
  - Not all genotoxins/carcinogens have DEREK alerts  
Only 46 of 92 GreenScreen HC genotoxicity positive compounds (50%) were DEREK alerting, despite the reliability of a GreenScreen positive result (above, c). Reassuringly DEREK "Plausible Chromosome Damage" alerts are more prevalent in GreenScreen HC positives (24%) than GreenScreen negatives (9.4%).
  - GreenScreen HC identifies non-alerting genotoxic compounds.  
Non-Alerting GreenScreen HC positive compounds included:



## Mode of Action

Pharmacological Class	Number
Phosphatidyl Kinase Inhibitor	17
Dopamine	10
Androgen	4
Adrenocorticoid	4
Cell Cycle	4
DNA Metabolism	4
Topoisomerase Inhibitor	4
Antiparasitic	3
Anticancer	3
Cyclic Nucleosides	3
Cytoskeleton and ECM	3
Hormone	3
Antimicrobial	2
Benzodiazepine	2
Ion Pump	2
Ca Channel	2
Lipid	2
Nitric Oxide	2
Protein Kinase	2
Tachykinin Antagonist	2
Adenosine Triphosphatase Inhibitor	1
Anticoagulant	1
DNA Alkylator	1
DNA Interactor	1
Enzyme Inhibitor	1
GABA	1
Multi-Drug Resistance	1
Na+ Channel	1
Neurotransmission	1
Opioid	1
Serotonin Receptor	1
Transcription Inhibitor	1
Vanilloid Agonist	1

Genotoxicity is detected in 92 mechanistically diverse pharmaceutical compounds.

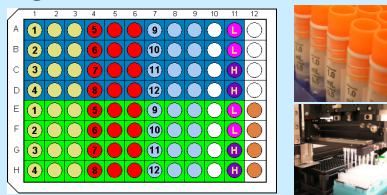
## Summary

- HTS is readily achievable: 240 compounds fully characterised per day (20 microplates)
- Compound consumption is low: 10  $\mu$ l of 10 mM DMSO stock per test – about 34  $\mu$ g of test article
- 100  $\mu$ M is in toxic range and limits interference from colour, autofluorescence and low solubility.
- Where data were available from carcinogenicity studies, GreenScreen positives were the most potent carcinogens. GreenScreen HC also demonstrated higher specificity than *in silico* methods.
- HTS screening from a fixed concentration reduces sensitivity, but high specificity is maintained. This is essential in early hazard assessment where mis-classification can kill compounds.
- Current collaboration with the National Institutes of Health - Chemical Genomics Center to reformat to 384 and 1536 well format. If successful this will be another step change in throughput for genotoxicity screening in whole cells.

This work is supported by Gentronix Limited. For contact details, enquires, a wealth of published papers or to purchase GreenScreen® kits - see [www.gentronix.co.uk](http://www.gentronix.co.uk)

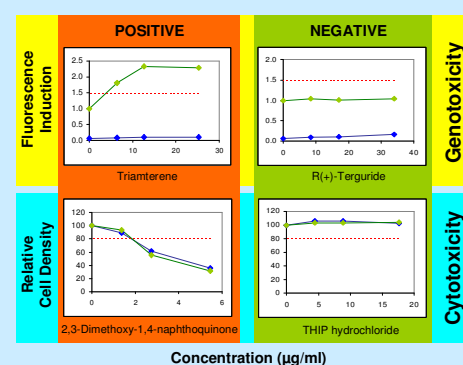


## Straightforward Protocol



- Pipette and dilute 12 test compounds to the microplate
- Add intra-plate QC: 2 doses of methylmethane sulfonate
- Add two cell strains: ■ Test and ■ Non-fluorescent Control cells
- Incubate (37 °C, 5% CO<sub>2</sub>) for 48 hours.
- Assess bulk fluorescence and absorbance in a microplate reader
- Automatic data processing using dedicated software

## GreenScreen HC Results



## Alternatives in HT Genotoxicity Screening?

Method	Description	Key Features
Ames II	Cut-down Ames assay using fewer strains	● Uses up to 12 x 384 well microplates per compound. Prokaryotic assay which suffers from low sensitivity.
VitoTox™ / SOSumuC	Bioluminescent / Colourimetric bacterial assays	● Essentially predictors of Ames test, therefore also low sensitivity. Applications more focussed on environmental monitoring.
Yeast Assays GreenScreen GC & DEL	GFP expression and Luminescence	● Incorporate eukaryotic endpoints, however highest sensitivity when used in conjunction with Ames screen.
<i>in vitro</i> Micronucleus	Flow cytometric methods	● Significant investment in equipment. Relatively low throughput. High prevalence for positive results for non-carcinogens.
GreenScreen HC	TK6 cells with GADD45a-GFP reporter	● High sensitivity and specificity. Low compound requirement. Automated data analysis. True HTS.