BlueScreen HC - A Luminescence Based, High-Throughput, In Vitro Genotoxicity Assay.

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- Protocol for the rapid and accurate detection of genotoxic liability in various test articles
- Test articles include pharmaceuticals, industrial chemicals & personal care products
- Genotoxicity & Cytotoxicity measured simultaneously using flash luminescence, absorbance and fluorescence

Introduction

The BlueScreen HC™ genotoxicity assay from Gentronix Ltd. uses a human-derived, p53-competent, TK6 cell line to host a luminescence-based reporter system that exploits the proper regulation of the GADD45a gene. GADD45a plays an important role in mediating the cellular response to genotoxic stress. The patented system incorporates complex regulatory elements to enable a faithful GADD45a response. The assay generates positive results for direct-acting mutagens and clastogens, as well compounds that act indirectly such as aneugens, and topoisomerase and polymerase inhibitors, which interfere with the processes of DNA replication, maintenance, repair and segregation. Importantly, correct negative results are produced for non-carcinogens, many of which give misleading positive results in other *in vitro* genotoxicity tests. In the BlueScreen HC S9 assay a metabolic activation protocol using rodent liver extract (S9) extends the range of compounds detected to include genotoxic metabolites. S9 contains cytochrome P450s and other enzymes that catalyse the detoxification of xenobiotic substances.

The BlueScreen HC assay utilizes a luciferase from the marine copepod *Gaussia princeps* to generate a luminescent output. Exposure to a genotoxic chemical causes increased expression of GADD45a and thus a dose dependent increase in the production of luciferase from the GADD45a reporter, which is naturally exported from the cell. The amount of luciferase produced by the cells is assessed by injection of a solution of the substrate, coelenterazine, resulting in a short-lived flash luminescence. In addition, reduced cell proliferation, a measure of cytotoxicity, is assessed either by changes in optical absorbance in the BlueScreen HC assay, or in fluorescence from a DNA stain in the BlueScreen HC S9 assay.

Materials and Methods

- ☐ BlueScreen HC and BlueScreen HC S9 reagent kits and cell line.
- ☐ BlueScreen HC – Black, clear flat-bottomed, sterile, 96-well microplates from Thermo Scientific Matrix.
- ☐ BlueScreen HC S9 – Black, solid round-bottomed, sterile, 96-well microplates from Greiner Bio-One.
- ☐ FLUOstar Omega microplate reader from BMG LABTECH.

Preparation of the Assay Microplates

The genetically modified reporter cells are maintained in an RPMI based culture medium and grown to a density between 0.5 and 1.2 x 10^6 cells/mL in preparation for the assay. 4 test articles (i.e. pure chemicals, compound mixtures or product formulations) are tested over 8 serial (2-fold) dilutions, together with the appropriate genotoxic positive control, in one 96-well microplate. After arraying the test compounds in duplicate, an equal volume (75 µL) of growing cells at a density of 2 x 10^6 cells/mL is added to each well in columns 1 to 11. Microplates are covered with a breathable membrane and incubated at 37°C, 5% CO_2 and 95% humidity without shaking. For studies without S9 metabolic activation the microplates are incubated for 48 hrs. For studies incorporating S9 metabolic activation, S9 is added to each well of the microplate containing cells at a final concentration of 1% v/v S9. After 3 hrs the cells are washed and then re-suspended in fresh recovery media for a further 45 hrs.

Fig. 1: BMG LABTECH's FLUOstar Omega multidetection microplate reader.

BlueScreen HC and BlueScreen HC S9 parameters

Microplates are shaken to re-suspend the cells before measurement. For the BlueScreen HC assay (without S9) the microplate wells are first assessed using absorbance measurements to determine cell density, then using flash luminescence to quantify luciferase expression. For the BlueScreen HC S9 assay, luciferase expression is quantified first, followed by cell lysis and assessment of cell density using thiazole orange which is fluorescent when bound to DNA. Reduced cell density compared to untreated cells provides a measure of cytotoxicity. Luminescence intensity, corrected for cell density, is proportional to the expression of GADD45a and thus the genotoxicity of the test article. Table 1 summarizes the differences between protocols in the absence and presence of S9.

FLUOstar Omega instrument parameters:

- **Absorbance:** 620 nm using 20 flashes per well.
- **Fluorescence:** Excitation 485 nm, emission 520 nm, optimal gain, top reading and 10 flashes per well.
- **Luminescence:** Measurements were made in "well mode", injecting, shaking and reading each well sequentially. 50 µL of a 2.5 µM solution of coelenterazine was injected into the well using the FLUOstar's in-built reagent injector at a speed of 260 µL/s. The microplate was then shaken for 1 s (500 rpm, orbital) immediately after which luminescence was recorded with an integration time of 5 s.

Table 1: BlueScreen HC assay protocols, run either in the absence or presence of S9 fraction.

	BlueScreen HC	*BlueScreen HC S9 with metabolic activation*
Metabolic activation	-	1% v/v S9 Fraction
Incubation Time	Exposure - 48 hours	Exposure - 3 hours / Recovery - 45 hours
Positive Control Compound	4-Nitroquinoline oxide (0.5 and 0.125 µg/mL)	Cyclophosphamide (25 and 5 µg/mL)
Luciferase Assay	Flash Luminescence	Flash Luminescence
Relative Cell Density Assessment	Optical Absorbance	DNA Stain Fluorescence
Microplate	Black, clear flat-bottomed, sterile, 96-well	Black, solid round-bottomed, sterile, 96-well

Data Handling

Absorbance, fluorescence and luminescence readings are exported directly into an Excel-based software template supplied by Gentronix. The software gives automated decisions (i.e. positive, negative), quantitative results (i.e. lowest effective concentration) and clear graphical dose response data for both genotoxicity and related cytotoxicity endpoints. Results for wells containing the test article are scaled relative to the vehicle (diluent) treated controls. Positive results are defined according to statistically derived threshold values for an increase in luminescence induction for genotoxicity and a decrease in relative cell density for cytotoxicity.

Results and Discussion

A range of compounds were tested with varying toxic properties.

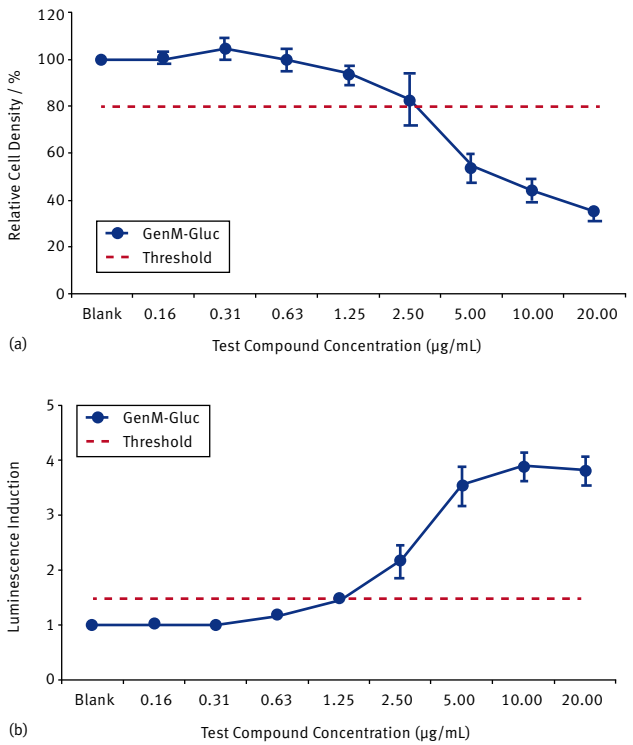

Fig. 2: BlueScreen HC S9 assay positive cytotoxicity (a) and genotoxicity (b) results for 20 µg/ml benzo[a]pyrene. Error bars show +/- 1 standard deviation based on 4 replicate analyses on separate microplates.

BlueScreen HC results are given in Table 2 and include 2 genotoxins (methyl methanesulfonate and 4-nitroquinoline oxide) and 1 cytotoxic non-genotoxin (2,4-dichlorophenol). BlueScreen HC S9 results are given in Figure 2 and Table 3 and include 3 pro-genotoxic chemicals that form genotoxic metabolites in the presence of S9.

Test Article	Concentration (µg/mL)	*Cytotoxicity*	*LEC (µg/mL)*	*Genotoxicity*	*LEC (µg/mL)*
Methyl Methanesulfonate	50	POSITIVE	12.5	POSITIVE	6.25
4-Nitroquinoline Oxide	1	POSITIVE	0.25	POSITIVE	0.13
2,4-Dichlorophenol	324	POSITIVE	40.5	NEGATIVE	-
Vehicle Control	-	NEGATIVE	-	NEGATIVE	-

Table 2: Cytotoxicity and genotoxicity results for 4 test articles, screened in the BlueScreen HC assay. LEC = Lowest effective concentration.

Test Article	Concentration (µg/mL)	*Cytotoxicity*	*LEC (µg/mL)*	*Genotoxicity*	*LEC (µg/mL)*
Aflatoxin	1.25	POSITIVE	0.08	POSITIVE	0.04
6-Aminochrysene	40	POSITIVE	5	POSITIVE	2.5
Benzo[a]pyrene	20	POSITIVE	5	POSITIVE	2.5
Vehicle Control	-	NEGATIVE	-	NEGATIVE	-

Table 3: Cytotoxicity and genotoxicity results for 4 test articles, screened in the BlueScreen HC S9 assay, incorporating exogenous metabolic activation for the detection of genotoxic metabolites. LEC = Lowest effective concentration.

LECs obtained for these well-characterised compounds were the same or within 1 serial dilution of historic controls and highly consistent between replicates.

Conclusion

The BlueScreen HC and BlueScreen HC S9 assays are shown here to be highly compatible with the BMG LABTECH FLUOstar Omega microplate reader making use of the 3 principal detection modes and the reagent injection system. Using the FLUOstar Omega the BlueScreen HC assay has been shown to be fast, accurate and reproducible in the detection and quantification of genotoxic liability of a broad range of chemicals with differing potencies and modes of action.

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