DB-ALM Protocol n° 132 : GreenScreen HC™ Genotoxicity Test

Genotoxicity/Mutagenicity

GreenScreen HC (Human Cells) is a fast, quantitative genotoxicity assay in vitro. The assay uses the DNA damage-inducible "*Growth Arrest and DNA Damage 45 alpha - Green Fluorescent Protein"* reporter gene, expressed in p53-competent TK6 cell line. The response to a chemical insult leads to an increase in green fluorescence. GreenScreen HC + S9 allows for a detection of genotoxic potential of the test compound with and without metabolic activation. (Available in reagent kit form)

Objective & Application

TYPE OF TESTING	:	Screening, Proposed as Part of Test Battery

Context of Use:

Currently used for non-regulatory purposes. GreenScreen HC (GSHC) provides an indication of a genotoxic potential of the test agent and/or its metabolites, formed in the presence of liver S9 fraction. Additionally, the assay provides an indication of the test agent's cytotoxicity through the measurement of either its effect on cell proliferation or cell viability.The International Conference on Harmonisation (ICH) recommends a standard test battery for genotoxicity assessment of pharmaceuticals, which includes the Ames bacterial reverse mutation test and one or more *in vitro* or *in vivo* tests in a mammalian system (S2(R1) Guideline; ICH, 2011). This test battery provides high sensitivity (the ability to predict *in vivo* genotoxicity*)*. However, the mammalian *in vitro* tests recommended by ICH (mouse lymphoma assay, *in vitro* micronucleus and chromosomal aberrations) are critcized for high number of false positives and low specificity. Their ability to predict the *in vivo* non-carcinogenicity is below 45%, according to Kirkland *et al.,* 2005; Snyder and Green, 2001; Matthews *et al*., 2006; Brambilla and Martelli, 2009. The GSHC assay for genotoxicity was developed to match the specificity level of an *in vivo* assay without compromising the sensitivity.The GSHC assay can be utilised as a screening tool or adjunct test within a test battery. Common applications refer to the assessment of toxic potency within risk assessment approach, drug discovery and development, compound prioritisation, lead compound testing and optimisation including pre-clinical safety assessment.

Applicability Domain:

The assay has been used with pharmaceuticals (drug discovery and development compounds), fine chemicals, cosmetics, pesticides, household products, can coatings, medical devices and plant extracts. The authors observe that the above list overlaps with the scope of REACH regulation (No 1907/2006; EC, 2006). Physicochemical properties of test agents can limit the assay's use. Specifically, the assay is sensitive to interference caused by highly light absorbing or highly fluorescent compounds. The majority of these are accommodated by internal assay checks and controls, the use of a control cell strain for data correction, and a fluorescence polarization data collection method that exploits the high fluorescence anisotropy of GFP.

Résumé

GADD45a (Growth Arrest and DNA Damage 45 alpha gene) is a DNA damage-inducible gene and the only GADD gene so far to respond to ionising radiation. Extensive peer-reviewed literature, particularly from the Fornace lab (e.g. Fornace *et al*., 1992), provides background on the biology of GADD45a and supports its usage as a risk indicator in genotoxicity testing. In fact, the induction of the transcription of GADD45a has been demonstrated in response to numerous different genotoxic stresses and in numerous different cell types such as, human lymphoblasts (Papathanasiou *et al*., 1991) and fibroblasts (Wang *et al*., 1999), various human tumour cell lines (Carrier *et al*., 1996), rodent cells *in vivo* (Hollander *et al*., 1999; O'Reilly *et al*., 2000) and rodent cell lines (Kastan *et al*., 1992; Fornace *et al*., 1989). The response is rapid, dose-dependent, highly conserved in mammalian cells and largely, though not exclusively, p53-dependent (Zhan, 2005).

The GreenScreen HC protocol indirectly measures genome damage and genotoxic stress by means of the *GADD45a-GFP* reporter gene (Hastwell *et al.*, 2006). Green Fluorescent Protein (GFP) is stable (half-life ~ 26 hours in mammalian cells; Corish and Tyler-Smith, 1999) and as such accumulates during the course of the assay to provide an assessment of the effect of the test compound on GADD45a transcription over the whole assay period (at least 1 cell cycle), when compared to the vehicle-treated cells.The use of a transcriptional reporter means that the assay relies upon the cellular response to DNA damage and genotoxic stress, as opposed to assessing the consequences of failed or erroneous repair (e.g. mutation).

The exposure routine is performed in microplates, so that the assay is scalable for high throughput studies (if needed), and the consumption of the test compounds and reagents is minimal. The assay can be further extended by including S9-mediated metabolic activation (Jagger and Tate *et al.*, 2009).

The GSHC assay is hosted in a human-derived cell line: TK6 cells are originally derived from a malignancy (Levy *et al.,* 1968; Skopek *et al.,* 1978) but retain p53 competency and are expected to exhibit cell cycle control and repair mechanisms akin to human *in vivo* (Zhang *et al*., 2007). These elements contribute favourably compared with existing assays hosted in p53-deficient cell lines derived from rodent malignancies. The TK6 cell line is also included in the OECD Test Gudeline 476 on " *In vitro* mammalian cell gene mutation test " (OECD, 1997).

The ICH-recommended test battery for the genotoxicity testing of pharmaceuticals defined in the S2B guideline (ICH, 1997) provides very high sensitivity in the identification of genotoxic carcinogens. However, this sensitivity is achieved to the detriment of the accurate identification of non-genotoxic non-carcinogens. This widely reported specificity issue (e.g. Kirkland *et al*., 2005), stemming overwhelmingly from the *in vitro* mammalian tests, results in misleading predictions of genotoxic hazard in animals. The need for new and/or improved *in vitro* mammalian tests has been identified and recognised by numerous international bodies, including the ICH (revised guideline S2(R1), 2011), ECVAM (Kirkland *et al*., 2007), ILSI-HESI (Thybaud *et al*., 2007a; Lynch *et al*., 2011), IWGT (Thybaud *et al.,* 2007b) and COLIPA (Pfuhler *et al*., 2010). The GreenScreen HC assay (Hastwell *et al*., 2006) was developed as a more accurate *in vitro* mammalian assay in response to this clear need.

Experimental Description

Endpoint and Endpoint Measurement:

CELL PROLIFERATION - measured by optical absorbance

CELL VIABILITY: measured by Propidium Iodide exclusion

DNA DAMAGE - disruption of genome integrity measured by the activity of DNA-damage- and aneugen-inducible GADD45a gene

GENE INDUCTION - measured by increase of GFP fluorescence

Endpoint Value:

LEC - Lowest Effective Concentration of a test compound

Experimental System(s):

Host cells: TK6 lymphoblastoid cell line (suspension cells)
Cell line origin: Human

Basic Procedure

GreenScreen HC assay utilises log phase cultures of 2 strains of the TK6 human lymphoblastoid suspension cell lines; a test strain bearing the active GADD45a-GFP reporter, and a non-fluorescent control strain bearing a GADD45a-GFP reporter inactivated by a 4bp deletion in the start sequence of the GFP gene (Hastwell *et al.,* 2006).

The protocol includes two related assays which can be performed separately or in parallel: **GSHC**, where the GFP accumulation is quantified by a microplate spectrofluorometric measurement and **GSHC+S9**, which includes metabolic activation of a test compound by S9 fraction, followed by a flow cytometric measurement of cell viability and GFP content. To reduce the interference caused by autofluorescence and opacity of the S9 preparations in GSHC+S9 assay, a flow cytometer is used to measure GFP fluorescence (Jagger & Tate *et al.*, 2009).

- **Assay A - GSHC**:
  During the assay, cells are maintained in a low autofluorescence growth medium supplemented with a serum-replacement (the assay is serum-free). Both types of cells and test compounds at 9 concentrations (usually 2-fold serial dilutions) are arrayed on a 96-well microplate (black walled, optically clear base, sterile), along with zero dose (vehicle-treated) controls and 2 different doses of positive control compound in duplicate.
  Positive control: Methyl methanesulfonate (MMS)
  The exposure of the assay cells to the test agent is continuous throughout the assay period. Endpoint

measurements are made following 24 and 48 hours ' test compound exposure. Two parameters are collected at each time point using a multi-mode microplate spectrophotometer: optical absorbance at ~ 620 nm (as an estimation of cell numbers and hence proliferative potential) and green fluorescence from the reporter GFP using FITC wavelengths (excitation at 485 nm and emission at 535 nm).

- **Assay B - GSHC+S9**:
  During the assay, cells are maintained in a low autofluorescence growth medium supplemented with either heat-inactivated donor horse serum or a serum-replacement (see below). Both types of cells and test compounds at 9 concentrations (usually 2-fold serial dilutions) are arrayed on a 96-well microplate (black walled, optically clear base, sterile), along with zero dose (vehicle-treated) controls and 2 different doses of positive control compound in duplicate.
  Positive control: Cyclophosphamide (CPA)
  The exposure of the assay cells to the test agent and S9 fraction is limited to 3 hours in a low autofluorescence growth medium supplemented with heat-inactivated donor horse serum. After the exposure period, the test agent and S9 fraction are removed from the assay. Following thorough washing steps, cells are then allowed to recover in a low autofluorescence growth medium supplemented with a serum-replacement during a further 45 hour incubation period. Endpoint measurements are made following the recovery period, 48 hours after the initiation of test compound exposure. Two parameters are collected using a flow cytometer equipped with an air-cooled 488-nm argon – ion laser and a 96-well microplate sampling adaptor, which enables sampling directly from the microplate wells (alternatively, a tube carousel can be utilised). Green fluorescence from reporter GFP is collected through the 530/30 nm (FL1) band-pass filter whilst red emission from propidium iodide (PI) is filtered through the 650LP nm (FL3) filter. Photomultiplier tube voltages are initially set using test cells (expressing GFP) or control cells (not expressing GFP), both stained with PI. Analysis is restricted to events representing living cells using appropriate logical gating relative to PI staining intensity.

Data Analysis/Prediction Model

The end result of the Green Screen assay is the qualitative (positive or negative) outcome of the assessment of the genotoxic potential for each compound tested.

The genotoxicity outcome of the assay, qualified by the cytotoxicity result, is governed by statistically defined thresholds for relative fluorescence induction. Different thresholds apply to the – S9 and +S9 assays due to the different instrument platforms used in data collection.

A positive genotoxicity result in **GSHC** assay is concluded if one compound dose produces a relative GFP induction greater than 1.5 of the vehicle-treated control and all of the acceptability criteria are fulfilled. A negative genotoxicity result is concluded where no compound doses produce a relative GFP induction greater than the 1.5 of the vehicle-treated control and all of the acceptability criteria are fulfilled. If a positive genotoxicity result is observed after 24 and/or 48 hours the compound is classified positive for genotoxicity. The LEC (Lowest Effective Concentration) for a positive genotoxicity result is the lowest test compound concentration that produces a relative GFP induction greater than the 1.5 of the vehicle-treated control threshold (determination of which is discussed in detail in the Data Analysis section of the Technical Description).

In the same way in **GSHC+S9** assay, a positive genotoxicity result is yielded if one compound dilution produces an increase in GFP induction greater than 1.3-fold above the vehicle-treated control. A negative genotoxicity result is concluded where no compound dilutions produce a relative GFP induction greater than the 1.3 of the vehicle-treated control threshold. Again, all acceptance criteria must be met for the assay to be considered valid.

Test Compounds and Results Summary

Assay A - GreenScreen HC

So far, data for 155 compounds have been published in the peer-reviewed literature for the **GreenScreen HC** protocol (without S9 fraction) in two dedicated studies (Hastwell *et al.*, 2006 and Hastwell *et al.*, 2009) and in parallel with **GSHC+S9** assay (Birrell *et al.,* 2010 - see Assay C below).

Subset I (Hastwell *et al.*, 2006)

- Mixed pharmaceuticals, industrial chemicals and intermediates of assorted known

genotoxic potential and classified mechanistically:

- Direct-acting genotoxins
- Topoisomerase inhibitors
- Aneugens
- Nucleotide / DNA synthesis inhibitors
- Reactive oxygen species generators

- Compounds with ' misleading predictions ' of *in vivo* genotoxicity/carcinogenicity outcomes by other *in vitro* mammalian genotoxicity tests
- *In vitro* genotoxicity negatives

GreenScreen HC assay on those 75 well-characterised genotoxic and non-genotoxic compounds yielded a sensitivity of 81% and a specificity of 95% for *in vivo* genotoxins, whilst these figures were 95% and 100% for genotoxic carcinogens.

Subset II (Hastwell *et al*., 2009)

- 75 Marketed pharmaceuticals with a variety of therapeutic indications including: Antineoplastic, Antiviral, Antifungal, Anthelmintic, Antibacterial, Antimalarial, Anti-inflammatory, Antirheumatic, Antihistaminic, Antidepressant etc.

GreenScreen HC assay was 88% predictive of *in vivo* genotoxicity data and 93% predictive of genotoxic carcinogenicity. No compounds were uniquely positive in the GSHC assay.

In the prediction of genotoxic carcinogenicity, the specificity of the assay is currently 95%, which is higher than that of other *in vitro* mammalian assays, and the sensitivity is 87.5%, which is higher than that of the Ames test (e.g. Hastwell *et al*., 2009). In the prediction of *in vivo* genotoxicity of 155 compounds, the specificity of the assay is 92% and the sensitivity is 85% (SOT, poster 1414, 2011). 1600 compounds have been tested by the assay developers (Knight *et al.,* 2009a, Knight *et al.,* 2009b). Data for > 4000 proprietary compounds have been generated by the assay`s users, though the majority of these compounds do not have a ' full set ' of comparative regulatory genotoxicity assay data.

Assay B - GreenScreen HC + S9 (Jagger and Tate *et al.*, 2009)

Data for 56 compounds were published from the **GreenScreen HC + S9** protocol (with S9 fraction), including:

- Expected pro-genotoxins
- Genotoxins (in the absence of metabolic activation)
- Non-genotoxins

Using a fixed set of conditions, GSHC+S9 identified 31/33 (94%) genotoxins or pro-genotoxins as positives, whilst 19/23 (83%) non-genotoxins gave negative results, two of which were only positive in one replicate and at concentrations of 5 mM or greater.

Assay C - Combined results from GreenScreen HC and **GreenScreen HC+ S9 assays**

A study of 60 ECVAM-recommended chemicals (Birrell *et al.,* 2010) for the assessment of new or improved genotoxicity tests (Kirkland *et al.,* 2008), included the following classes:

- Ames-positive *in vivo* genotoxins
- *in vivo* genotoxins negative or equivocal in Ames test
- Non-carcinogens with negative *in vivo* genotoxicity data
- Non-carcinogens with no genotoxicity data *in vivo*
- Non-genotoxic carcinogens
- Compounds with equivocal predictions of *in vivo* genotoxicity / carcinogenicity outcomes by other *in vitro* mammalian genotoxicity tests

All compounds were subjected to both **GreenScreen HC** and **GreenScreen HC + S9** assays in triplicate. 18/20 (90%) genotoxins were reproducibly positive in GSHC (± S9), 22/23

(96%) expected non-genotoxins were reproducibly negative in GSHC (± S9) and 13/17 (76%) expected negatives with misleading data in other assays were reproducibly negative in GSHC (± S9)

Modifications of the Method

The original method as published by Hastwell *et al*. (2006) used an assay medium incorporating heat-inactivated donor horse serum. That method was subsequently adapted to operate in serum-free assay medium, using a defined serum replacement supplement in place of the serum. Subsequent published validation studies and compound datasets were all from studies performed using the serum-free method, which is descried in this Protocol. Reasons for the modification were threefold:

1. Reduction of animal-derived reagent usage in the assay method.
2. Reduction of background contribution caused by serum characteristics that can impinge on optical endpoints.
3. Reduction of the variability of background contribution caused by between-batch and between-supplier differences in serum.

Discussion

A number of studies in the last decade have considered the utility of the current ICH-recommended battery of *in vitro* genotoxicity tests, particularly within the domain of pharmaceutical safety assessment (e.g. Snyder and Green, 2001; Brambilla and Martelli, 2009; Kirkland *et al*., 2005). All of these point to the conclusion that the incidence of misleading *in vitro* data (in this context, positive data for non-carcinogens or non-genotoxic carcinogens) is unacceptably high. The issue has been acknowledged by a number of bodies/agencies and there is general agreement that existing methods need improving through protocol and guideline revisions (e.g. S2(R1); ICH, 2011), and/or new, more accurate tests need to be developed. The GreenScreen HC GADD45a-GFP assay fits into the latter category for the following reasons:

- It is hosted in a human-derived cell line
- The host cell line is wild-type for p53
- GADD45a has been demonstrated to respond to all types of genotoxic stress
- The microplate format and rapid assay timeframe (48 hours) allow for relatively high throughput with only low sample requirement
- Validation study data repeatedly illustrate the assay ' s high accuracy for prediction of both *in vivo* genotoxicity and genotoxic carcinogenicity
- To perform the protocol, equipment routinely available in most toxicology or cell-based assay labs is sufficient.

The assay authors (see Procedure Details section for contact information) can provide training in the assay that begins with a full description of the assay protocol, data collection, data handling and results interpretation. This is followed by a thorough demonstration of the practical steps in the test method and then observation of the new users performing the method. The transfer training period allows for 2 complete assay runs with data collection. Instruments are also set up for the assay as necessary. Users who have undergone the test method training are then recommended to self-certify by re-testing 4 compounds to achieve reproducible and repeatable data that meet all of the acceptance criteria.

Throughput: up to 120 compounds per week, performed manually.

Elements of automation can be employed to increase assay throughput.

Status

Known Laboratory Use:

The GreenScreen HC GADD45a-GFP assay reagent kits have been used in > 25 different laboratories around the world, ranging from global pharmaceutical companies to small biotech companies and Contract Research Organisations.

GreenScreen HC kit and training services are available from Gentronix Ltd (Manchester, UK) and BioReliance Corporation (Rockville, MD, USA).

> 100 pharmaceutical, biotech and fine chemicals companies in 17 countries have used the GreenScreen

HC assay data as part of their genotoxicity profiling strategy (**www.gentronix.co.uk**).

Participation in Evaluation Studies:

Marketed pharmaceuticals assessment (Hastwell *et al*., 2009) and the assement of ECVAM - recommended (Kirkland *et al.*, 2008) list of genotoxins and non-genotoxins (Birrell *et al*., 2010). The results of both studies are discussed in detail in the Test compounds and Results Summary section.

Participation in Validation Studies:

Development and validation of the GreenScreen HC GADD45a-GFP assay has followed the ECVAM's modular approach to principles of test validity (Hartung *et al*., 2004). However, all of the studies have been performed independently of ECVAM.

- **Assay A - GSHC:**

  Test definition & Within-laboratory variability - Hastwell *et al*., 2006
  The first publication of the assay provided GSHC methodology and results from a 75 compound training set. Quadruplicate testing of the training set yielded the following within-laboratory reproducibility information:

  - 95% sensitivity and 100% specificity for genotoxic carcinogens
  - 74 of 75 compounds gave the same assay outcome in each of the 4 replicate tests

  Transferability & Between-laboratory variability - Billinton *et al*., 2008
  The GSHC assay was transferred to 3 other laboratories and all 4 labs undertook a study of 16 coded compounds tested in quadruplicate. Minor protocol modifications were made as a result of the study, acceptance criteria were tested and overall concordance of trial results was 92.5%.

- **Assay B - GSHC+S9:**

  Test definition & Within-laboratory variability - Jagger, Tate *et al*., 2009
  Training set of 56 compounds was tested. Triplicate testing of the training set yielded the following within-laboratory reproducibility information:

  - 20 of 25 pro-genotoxins were correctly identified with the S9 protocol
  - 21 of 23 non-genotoxins were correctly identified with the S9 protocol
  - 48 of 56 compounds gave the same assay outcome in each of the 3 replicate tests

  Transferability, Protocol refinement & Between-laboratory variability - Billinton *et al*., 2010
  The GSHC+S9 protocol was transferred to 3 other laboratories and all 4 labs undertook a study of 8 compounds tested in triplicate. The method was refined and the study yielded 100% agreement in results between all 4 labs.

Regulatory Acceptance:

None as yet.

The UK Committee on Mutagenicity has recently produced " GUIDANCE ON A STRATEGY FOR GENOTOXICITY TESTING OF CHEMICAL SUBSTANCES " , in which it was recognised that the predominant use of high throughput screening tests is an aid in prioritisation of compounds for development undertaken by industry, and agreed that the *GADD45a* -GFP assay is most suited as part of a battery of high throughput screens (IACOM, 2011).

Health and Safety Issues

General Precautions

All work should be carried out in a Class II safety cabinet to avoid assay culture contamination and to avoid exposure to toxic analytes and assay control compounds. All test and standard compounds should be handled in a fume hood or vented safety cabinet. All work should be carried out using appropriate personal protection equipment, and should be carried out following good laboratory practices.

Methyl methanesulfonate (MMS): toxic; may cause cancer.
Cyclophosphamide (CPA): toxic if swallowed; may cause cancer.

Propidium iodide (PI): irritant.

Chemicals without safety information and/or coded are potentially harmful and should handled with maximum care.

The host cell strains used in the GreenScreen HC assay are genetically modified. The assay must therefore only be performed in a laboratory registered for the use of Genetically Modified Organisms, and in accordance with all local and national regulations relevant to the testing laboratory. Under ACDP Classification, the host organisms have been classified as Hazard Group 1 (lowest category), Unlikely to Cause Human Disease, as defined by the UK ' s Health and Safety Executive (Contained Use Regulations, 2000).

MSDS Information

Example MSDSs for standard compounds are downloadable from suppliers such as Sigma-Aldrich:

MMS: **http://www.sigmaaldrich.com/catalog/product/aldrich/129925**
CPA: **http://www.sigmaaldrich.com/catalog/product/sigma/c0768**
PI: **http://www.sigmaaldrich.com/catalog/product/sigma/p4170**

Abbreviations and Definitions

3R's:	Replacement, Reduction and Refinement of animal experimentation
COLIPA:	The European Cosmetics Association
ECVAM:	European Centre for the Validation of Alternative Methods. As from 2011, ECVAM has been established as the European Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM), hosted by the Joint Research Centre , Institute for Health and Consumer Protection.
EGFP	Enhanced Green Fluorescent Protein
FITC:	Fluorescein Isothiocyanate
GSHC:	GreenScreen Human Cells
GFP:	Green Fluorescent Protein
GADD:	Growth Arrest and DNA Damage
ICH:	International Conference on Harmonisation
ILSI-HESI:	International Life Sciences Institute, Health and Environmental Sciences Institute
IWGT:	International Workshop on Genotoxicity Tests
LEC:	Lowest Effective Concentration
PI:	Propidium Iodide
REACH:	Registration, Evaluation, Authorisation and Restriction of Chemicals
S9:	Supernatant fraction from liver homogenate contaning cytosol and microsomes

Last update: 28 June 2011

PROCEDURE DETAILS, 28 June 2011

GreenScreen HC™ Genotoxicity Test
DB-ALM Protocol n° 132

Available in reagent kit form, contract testing service and site license.

Latest SOP`s:
GreenScreen HC Genotoxicity and Cytotoxicity Assay Protocol (v3.3), March 2008
GreenScreen HC Genotoxicity Assay: S9 Protocol (v2.2), July 2008

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Materials and Preparations

Cell or Test System

Original host cells: TK6; lymphoblastoid; human-derived (ECACC Cat. No. 95111735; ATCC Number CRL-8015)

GreenScreen HC cells:
GenM-C01 – TK6 GreenScreen HC control cell strain
GenM-T01 – TK6 GreenScreen HC test cell strain
Cell strains are available from **Gentronix Ltd** as part of the assay reagent kits.

Equipment

Fixed Equipment

- Microplate reader capable of fluorescence readout using FITC wavelengths (excitation at 485 nm and emission at 535 nm) and absorbance reading at ~ 620 nm. (Gentronix can provide advice with regards to suitable models)
- For GSHC+S9 protocol, a flow cytometer with 488 nm argon-ion laser, green and red fluorescence channels and either a microplate sampling adaptor or a tube carousel.
- Haemocytometer or other equipment for accurately counting cell culture density.
- An incubator capable of maintaining +37 ˚ C, 5% CO_2 and 95% humidity.
- Class II safety cabinet.
- Preferably the capability to store frozen cell aliquots in cryovials in liquid nitrogen (i.e. an appropriate liquid nitrogen dewar); alternatively, a -80 ˚ C ultra-low temperature freezer for shorter term storage.
- 8- or 12- channel pipette with 100 µ l capacity, capable of accurately pipetting 75 µ l aliquots.
- Bench-top centrifuge with swinging bucket type rotor (max. required for the protocols ~ 340 x *g*)
    - Rotor suitable for microplate centrifugation required for GSHC+S9 protocol
- Water bath capable of maintaining 37 ˚ C.
- A flat-bed microplate shaker, suitable for resuspending cells after microplate centrifugation.

Consumables

- Black, clear-bottom, 96-well, sterile microplates (ThermoFisher Matrix ScreenMates, catalogue no. 4929 are routinely used)

- For GSHC+S9 protocol, round / U-bottomed (*not* conical), clear polystyrene, 96-well, sterile microplates (e.g. ThermoFisher Matrix ScreenMates, catalogue no. 4912 are routinely used).
- Plastic reagent troughs for pipetting (e.g. Corning Costar 4870, 50 ml reagent reservoirs)
- Breathable microplate sealing membranes (e.g. Diversified Biotech, Breathe-Easy sealing membrane).
- Cell culture flasks (with canted neck) - typically 75 or 225 cm ^{2}

Media, Reagents, Sera, others

Green Screen HC kits can be purchased from Gentronix Ltd.

GreenScreen HC kit contents (for 20 test compounds)

- Cell reagents - 2x GenM-C01 and 2x GenM-T01 cell strain aliquots (1 ml per vial), mycoplasma-free
- Assay medium – 5x aliquots of 9 ml *or* 1x aliquot of 44 ml (for HC-20B format)
- Assay medium supplement – 1x aliquot of 12 ml
- GreenScreen HC S9 kit contents (for 20 test compounds):
- Cell reagents - 2x GenM-C01 and 2x GenM-T01 cell strain aliquots (1 ml per vial)
- Co-factor solution – 5x aliquots of 1.8 ml (0.5 mM beta-NADP, 2.5 mM D-glucose-6-phosphate)
- Exposure medium – 5x aliquots of 10.8 ml
- Recovery medium – 5x aliquots of 12.6 ml
- Recovery medium supplement – 1x aliquot of 9 ml

Routine cell culture reagents
Note: Store media and components in accordance with the manufacturer's instructions.

- RPMI 1640 + 2 mM stable glutamine + 25 mM HEPES
- Heat-inactivated donor horse serum (e.g. Sigma-Aldrich, Lonza - sourced from US herds)
- Sodium pyruvate
- Penicillin / Streptomycin
- Hygromycin B

Additional required reagents

- Dimethyl sulfoxide
- Phosphate buffered saline *without* calcium, magnesium or phenol red.
- Sterile distilled water
- For GSHC+S9 - Cyclophosphamide monohydrate
- For GSHC+S9 - propidium iodide
- For GSHC+S9 - rat liver S9 fraction (e.g. Molecular Toxicology, Inc., catalogue number 11-101, 1 ml aliquots of Aroclor 1254-induced male Sprague Dawley rat liver S9 in 0.15 M KCl; store at -80 ° C)

Preparations

Media and Endpoint Assay Solutions

It is recommended that all reagents are purchased as sterile filtered, cell culture tested solutions. If reagents are bought as powders they should be dissolved in distilled, sterile water to the appropriate stock concentration. These solutions should then be filter sterilised and stored at the appropriate temperature.

All reagents and cell cultures must be handled under aseptic conditions.

To heat inactivate horse serum (if not already heat inactivated when purchased) - heat to 56 ° C for 30 minutes. Hygromycin B can be purchased as a pre-filtered, cell culture tested solution. If purchased as a powder, resuspend in sterile distilled water at a concentration of 50 mg/ml. Filter sterilise and store at 2 - 8 ° C.

Routine Culture Medium:
Note: Reagents should all fit into a standard 500 ml RPMI 1640 medium bottle. The prepared media should be labelled with its preparation date and a two week expiry date. It should be stored at 2 - 8 ° C,

out of direct sunlight.

To make routine culture medium, combine the reagents listed in the table below:

Reagent	Stock Concentration	Final Concentration	Volume (ml)
RPMI 1640 + stable glutamine	-	-	500
Sodium Pyruvate	100 mM	1.8 mM	10.4
Hygromycin B	50 mg/ml	200 µg/ml	2.3
Penicillin/Streptomycin	5,000 IU/ml / 5,000 µ g/ml	50 IU/ml / 50 µ g/ml	5.8
Heat-inactivated horse serum	100%	10%	57

For GSHC:

Complete Assay Medium:

Assay medium is combined with assay medium supplement to produce a cell culture medium with low autofluorescence specially formulated by Gentronix Ltd. for the GreenScreen HC assay. To make the complete assay medium combine the two reagents in the ratio listed in the table below. It is recommended that the volume of medium supplement required is added directly to the bottle of stock assay medium. When the reagents are combined the assay medium has a medium supplement concentration of 20% v/v. Complete assay medium should be labelled with its preparation date and has a shelf life of 45 days after addition of supplement. Media should be stored at 2 - 8 ° C, out of direct sunlight.

For the HC-20 kit, aliquots of 11.25 ml of complete assay medium are made, sufficient for individual assay microplates (4 compound tests per plate). For HC-20B kits, up to 55 ml of complete Assay Medium is made, sufficient for 5 microplates (20 compound tests).

GreenScreen HC Kit	Reagent	Volume (ml)
HC-20	**GS-HC-AM Stock Medium**	9
	GS-HC-AM Medium Supplement	2.25

HC-20B	**GS-HC-AM Stock Medium**	44
	GS-HC-AM Medium Supplement	11

For GSHC+S9:

Complete Exposure Medium:

Exposure medium is combined with heat inactivated horse serum to produce the medium used in the compound and S9 exposure period of the GreenScreen HC assay. To make the complete exposure medium combine the two reagents in the ratio listed in the table below. It is recommended that the volume of heat inactivated horse serum required is added directly to the bottle of exposure medium. When the reagents are combined, the complete exposure medium has a horse serum concentration of 10% v/v. Complete exposure medium should be labelled with its preparation date and has a shelf-life of 14 days after addition of serum. Medium should be stored at 2 - 8 ° C, out of direct sunlight.

For the HCS9-20 kit, aliquots of 12 ml of complete exposure medium are sufficient for an individual microplate (4 compound tests per plate).

GreenScreen HC Kit	Reagent	Volume (ml)
HCS9-20	**Exposure medium**	10.8
	Heat inactivated horse serum	1.2

Complete Recovery Medium:

Recovery medium is combined with GreenScreen HC/S9 recovery medium supplement to produce a complete recovery medium. To make the complete recovery medium combine the two reagents in the ratio listed in the table below. It is recommended that the recovery medium supplement is added directly to the bottle of recovery medium. When the reagents are combined, the complete recovery medium has a concentration of 10% v/v recovery medium supplement. Complete recovery medium should be labelled with its preparation date and has a shelf life of 45 days after addition of supplement. Medium should be stored at 2 - 8 ° C, out of direct sunlight.

For the HCS9-20 kit, aliquots of 14 ml of complete recovery medium are made, sufficient for an individual microplate (4 compound tests per plate).

GreenScreen HC Kit	Reagent	Volume (ml)
HCS9-20	GS-HC/S9-RM	12.6
	GreenScreen HC/S9 Recovery Medium Supplement	1.4

S9 metabolic activation:

Vials of S9 fraction and co-factor solution need to be removed from the ultra-low (-80 ° C) freezer up to an hour prior to intended usage and allowed to defrost on ice.
See page 22 (Microplate set-up stage 2) in the Method > Test Material Exposure Procedures section for further instructions.

Test Compounds

Diluent:

Typically, 2% DMSO (v/v) in sterile water.
100 ml of diluent should be prepared per run of 4 microplates such that the same stock diluent can be used to prepare and dilute all the standard and test compounds. For example, to prepare 100 ml of 2% DMSO, combine 2 ml of DMSO with 98 ml of sterile water and mix thoroughly.

For GSHC:
All concentrations of standard and test compounds are stated as made up by the operator. Using the standard dilution protocol described here, all concentrations are halved on the plate when a sample volume of 75 µl is combined with 75 µl of cell culture.

All standard and test chemicals should be freshly prepared shortly before the GreenScreen HC assay plate is set up.

Test Compounds:
The final concentration of each test compound must be in a solution that matches the diluent used, typically 2% DMSO in sterile water, such that the diluent solvent itself is not diluted across the plate.
An initial concentration of 2 mM or 1 mg/ml (whichever is lowest) is recommended (equating to 1 mM or 500 µg/ml of test compound on the microplate). It is desirable that the test compound is fully soluble at the top concentration tested. A minimum of 400 µl of each test compound is required per plate.

***For GSHC+S9*:**

Using the standard dilution protocol described here, all concentrations of test and control compounds are diluted 2.5 fold on the microplate when a sample volume of 60 µl is combined with 75 µl of cell culture and 15 µl of S9 mix.
All test chemicals should be freshly prepared shortly before the GreenScreen HC assay plate is set up.

Test Compounds:
As above, the final concentration of each test compound must be in a solution that matches the diluent used, typically 2% DMSO in sterile water.
An initial concentration of 2.5 mM or 1.25 mg/ml (whichever is lowest) is recommended (equating to 1 mM or 500 µg/ml of test compound on the microplate). It is desirable that the test compound is fully soluble at the top concentration tested. A minimum of 400 µl of each test compound is required per plate. Since the on-plate dilution is 2.5 fold all compound stocks must be at 2.5 fold greater than desired test concentration in 2% DMSO.

The recommended method to prepare solutions of test compounds is as follows:

In both GSHC and GSHC+S9:

• For compounds with high aqueous solubility - dissolve directly in aqueous diluent (i.e. 2% DMSO) and further dilute with diluent as necessary.

• For compounds of limited aqueous solubility - dissolve in 100% DMSO, dilute as

necessary in 100% DMSO, and then add 20 µl of the DMSO stock standard to 980 µl of sterile water to produce a test solution in 2% v/v DMSO. If the compound precipitates from solution when the DMSO standard is added to water, the original DMSO stock standard can be diluted further with 100% DMSO. The 20 µl + 980 µl water dilution step is then repeated to produce a fresh test standard.

The microplate layout is designed for the assay of 4 compounds per microplate. If fewer than 4 compounds are to be tested on a microplate, the " missing " compounds must be substituted i.e. wells must not be left empty. Alternatives for the compound substitute include running a duplicate of one of the other test compounds or using diluent alone as a test compound (effectively running a blank).

Positive Control(s)

For GSHC:

The methyl methanesulfonate (MMS) positive controls are prepared in diluent to the following concentrations:

- Standard 1 - MMS HIGH = 100 µg/ml
- Standard 2 - MMS LOW = 20 µg/ml

A minimum of 300 µl of each MMS control is required per plate. The following suggested dilutions produce 2 - 2.5 ml for each standard.

MMS should be freshly prepared from 100% stock solutions before use.

When preparing MMS standards ensure thorough mixing at each dilution stage by repeated aspiration and dispensing of the measured aliquot in the diluent, and/or by vortexing the solution in a sealed vial.

Dilution from MMS stock:

- Stock dilution: Add 5 µl of 100% MMS to 1300 µl diluent = 5 mg/ml MMS
- MMS HIGH: Dilute 50 µl of 5 mg/ml MMS with 2450 µl diluent = 100 µg/ml MMS
- MMS LOW: Dilute 400 µl of MMS HIGH with 1600 µl diluent = 20 µg/ml MMS

For GSHC+S9:

Cyclophosphamide (CPA) is used as the positive control for genotoxicity in this assay and is prepared in diluent to the following concentrations:

- Standard 1 - CPA HIGH = 62.5 µg/ml to give a final concentration of 25 µg/ml
- Standard 2 - CPA LOW = 12.5 µg/ml to give a final concentration of 5 µg/ml

The following suggested dilutions produce sufficient solution for 2 microplates. When preparing CPA standards ensure thorough mixing at each dilution stage by repeated aspiration and dispensing of the measured aliquot in the diluent, and/or by vortexing the solution in a sealed vial.

Prepare a stock solution of 1 mg/ml CPA in diluent.

Dilution from CPA stock:

- CPA HIGH: Dilute 75 µl of 1 mg/ml CPA with 1125 µl diluent = 62.5 µg/ml CPA
- CPA LOW: Dilute 15 µl of 1 mg/ml CPA with 1185 µl diluent = 12.5 µg/ml CPA

Batch aliquots of these solutions can be prepared for use in the assay as the compound is stable if kept frozen at -20 ° C. Do not thaw and then re-freeze.

Negative Control(s)

n/a

Method

Routine Culture Procedure

Cell lines:

Two cell lines are provided; a control cell line (GenM-C01) and a test cell line (GenM-T01). These are both required to perform a GreenScreen HC assay and should be maintained as separate cultures. Cells must be maintaned under sterile conditions and before passaging or using cells in any assay, must be examined microscopically for the presence of any contamination.

Thawing frozen cells:
Take a vial of GenM-C01 and a vial of GenM-T01 from cold storage and thaw quickly at 37 ˚ C in a water bath, until the frozen core can be dislodged. Tip the entire contents of each vial into a separate sterile 75 cm ² culture flask.

Over a period of 2 min, add 50 ml of pre-warmed (37 ˚ C) culture medium to the cells. Once resuspended, cells should be cultured at 37 ° C, 5% CO2, 95% humidity for 2 to 3 days (the density achieved in this time should be between 2 x 10 ^{5} and 2 x 10 ^{6} cells per ml). Harvest cells by centrifugation at ~ 340 x *g*, decant the supernatant and resuspend the cells in 50 ml pre-warmed culture medium at an appropriate density for the number of days they are to be passaged as described below.

Routine culturing (passaging):
Cell cultures are maintained in standard 75 or 225 cm ^{2} cell culture flasks utilising standard RPMI-1640 culture medium supplemented with stable L-glutamine, heat-inactivated donor horse serum and sodium pyruvate, incubated in a static CO_2 incubator set at 37 ° C (± 1 ° C), 5% (± 0.5%) CO_2 and 95% (± 3%) relative humidity.

Maintain cell lines in log phase by passaging every 1 to 4 days. Measure cell culture densities and dilute aliquots with fresh pre-warmed culture medium to a final cell titre of 0.15 - 5 × 10 ^{5} cells/ml depending on requirements (see table below).

Passage duration (days)	Starting cell concentration (cells/ml)
1	5 × 10 ^{5}
2	1.5 × 10 ^{5}
3	5 × 10 ^{4}
4	1.5 × 10 ^{4}

Recommended passage dilutions to achieve a cell concentration of approx. 1 x 10 ^{6} cells / ml on the day assays are commenced.

The assay requires cells to be in exponential growth phase; therefore cultures should have achieved a density of between 5 x 10 ^{5} cells/ml and 1.2 x 10 ^{6} cells/ml before they can be used in the assay. Cell cultures with a density outside of these limits should not be used and instead should be passaged for a subsequent day using the guidance above to achieve the required density.

Each assay plate run will require approximately 5 ml of each of the two cell line cultures at a starting concentration of 2 × 10 ^{6} cells/ml. Users are recommended to take account of this requirement when planning and performing passaging, such that sufficient culture will be available for both assay plates and for future passaging to maintain the cell lines.

Before passaging or using cells in an assay, examine cells microscopically for the presence of any contamination.

Before taking cell counts for passaging or assays, shake cultures gently to avoid excessive frothing which can have a detrimental effect on cell viability.

Cells should initially be passaged for 2 weeks in the presence of Hygromycin B before the cultures are used in assays.

Cultures can be maintained in passage for up to 3 months, at which time new cultures should be established from frozen cell stocks.

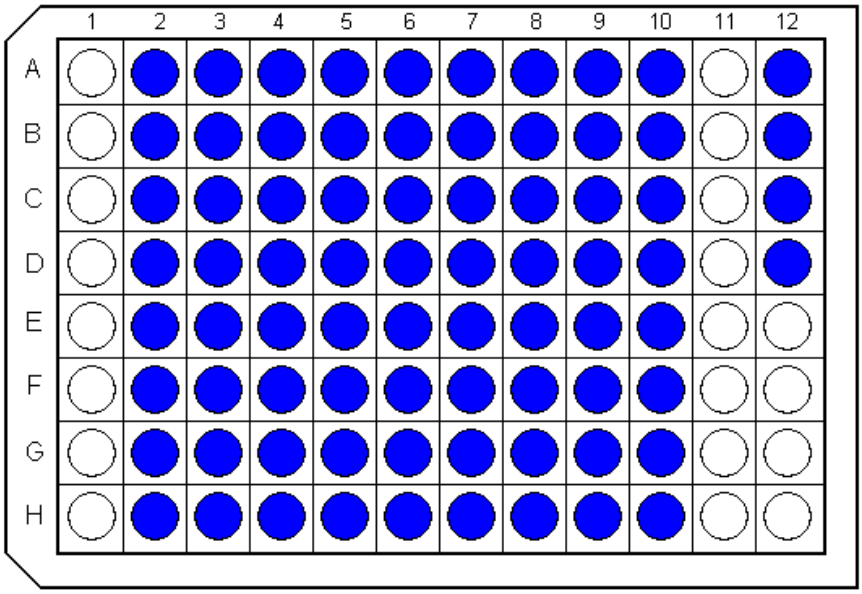
Test Material Exposure Procedures

For GSHC assay - go to next step
For GSHC+S9 assay - go to page 20

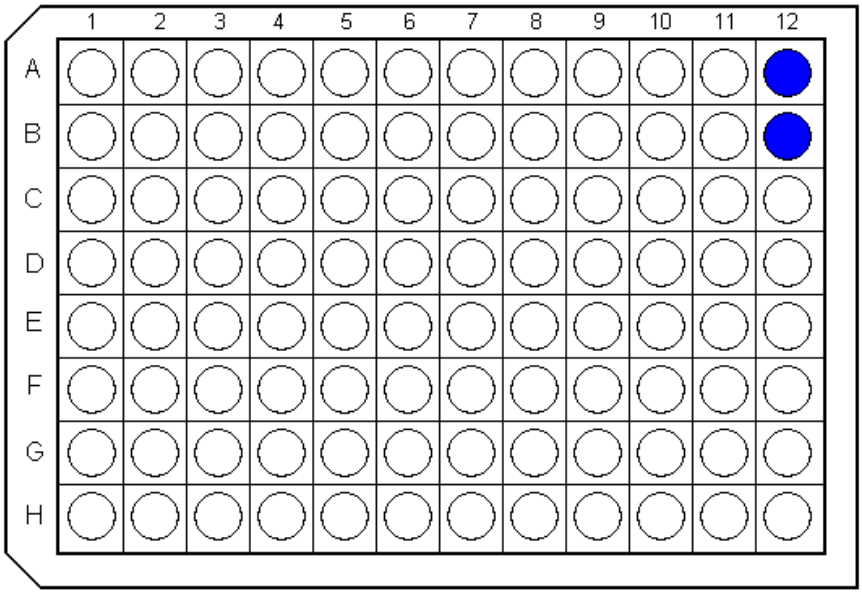
GSHC Assay:

Microplate set-up Stage 1

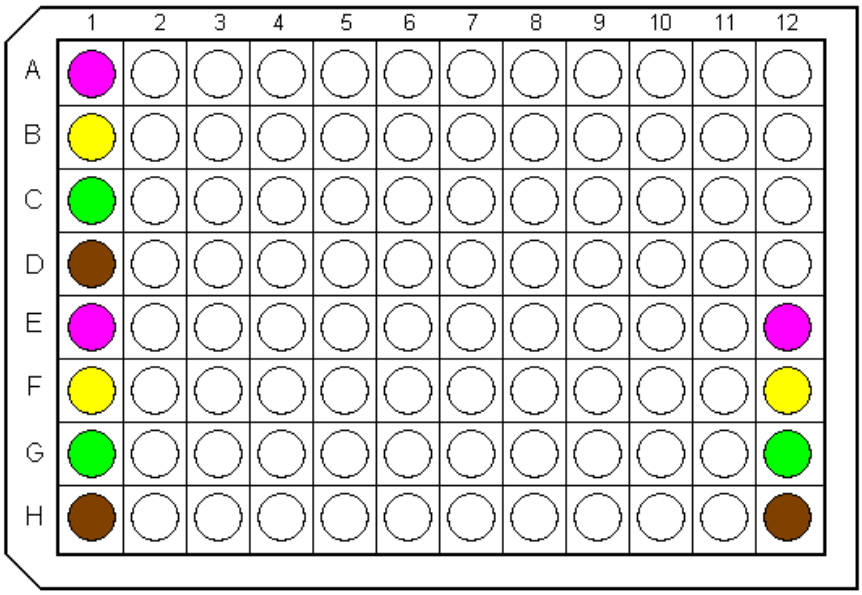
1. **Dispensing the test diluent**. [Use an **8-channel** pipette dispensing **75 µl**]
   - Omitting wells in columns 1 and 11 and E12 to H12, dispense **75 µl** of diluent into all other wells column-wise.



   - Dispense an additional **75 µl** of diluent into wells **A12** and **B12**.

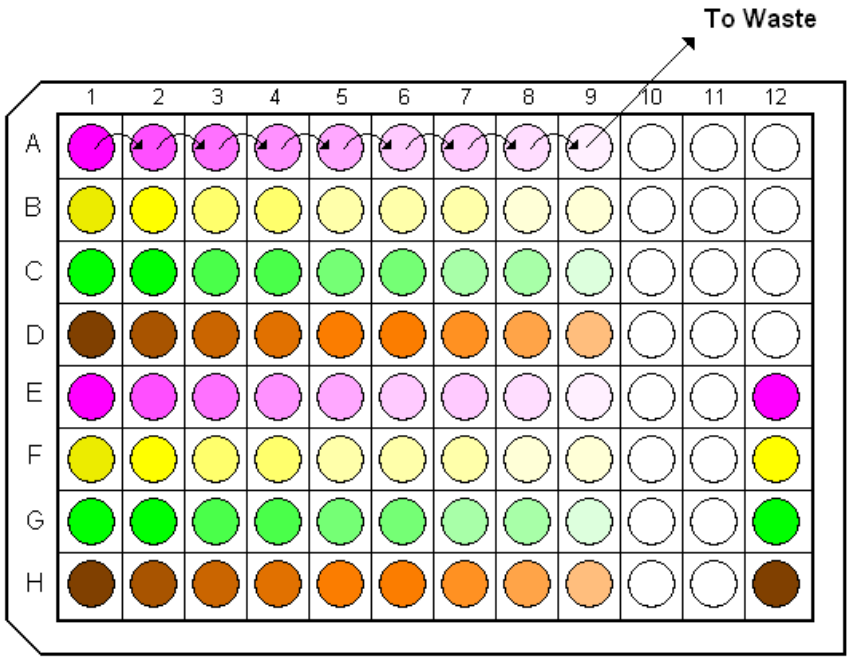


2. **Dispensing the test compounds**. [Use a **single-channel** pipette dispensing **150 µl and 75 µl**]
   - Dispense **150 µl** of test compound 1 into wells **A1** and **E1**, and **75 µl** into **E12**.
   - Dispense **150 µl** of test compound 2 into wells **B1** and **F1**, and **75 µl** into **F12**.
   - Dispense **150 µl** of test compound 3 into wells **C1** and **G1**, and **75 µl** into **G12**.
   - Dispense **150 µl** of test compound 4 into wells **D1** and **H1**, and **75 µl** into **H12**.



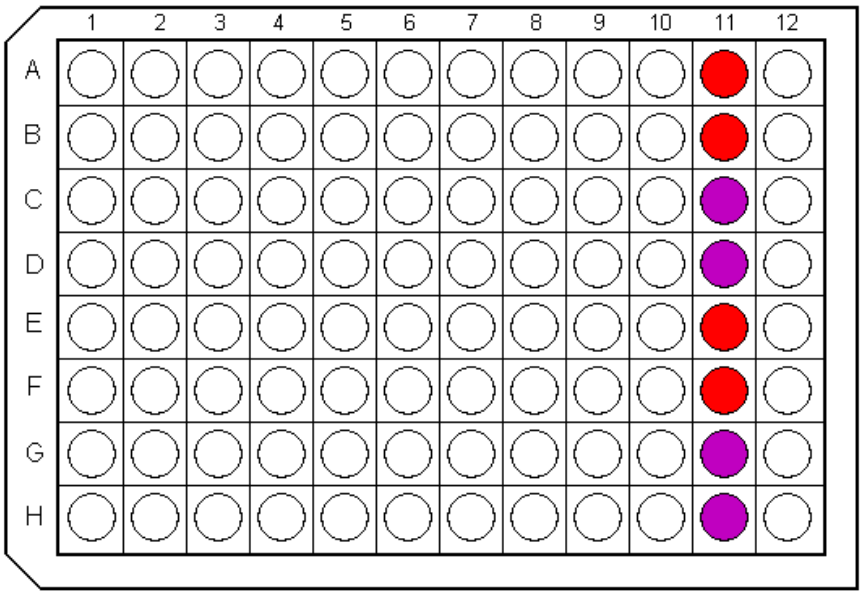
3. Serially diluting the test compounds. [Use an **8-channel** pipette dispensing **75 µl**]

- Aspirate **75 µl** from all wells in column 1 and dispense into column 2. *When dispensing into these wells mix the contents by repeated aspiration / dispense, or by using the mix feature common to many electronic auto-pipettes*
- Using the same tips repeat the entire process for the next column to the right, starting with aspiration of **75 µl** from column 2.
- Repeat this procedure up to and including column 9. After mixing the well contents in column 9, aspirate **75 µl** and discard to waste.



4. Dispensing the standard control compounds. [Use a **single-channel** pipette dispensing **75 µl**]

- Dispense **75 µl** of the **MMS LOW** control into wells **A11, B11, E11** and **F11**
- Dispense **75 µl** of the **MMS HIGH** control into wells **C11, D11, G11** and **H11**



5. Preparation of cell lines for addition to the microplate:

For each assay plate, prepare 5 ml suspensions of GenM-C01 and GenM-T01 cells at a density of 2 × 10^6 cells/ml in complete assay medium as follows:

Before taking cell counts shake cultures GENTLY to resuspend the cells.

Calculate the cell density from the routine cultures of GenM-C01 and GenM-T01 cells.

Note: For use in the assay the cell culture densities must be between 5 x 10^5 cells/ml and 1.2 x 10^6 cells/ml. If the cell culture density of either GenM-C01 or GenM-T01 lies outside of this range the assay should not be run and the cells should be passaged for a subsequent day using the guidance provided.

A total of 1 × 10^7 cells (i.e. 5 ml at 2 x 10^6 cells per ml) of each cell line are required per assay plate. Use the following equation to calculate the volume (V) of routine cell culture required to prepare cells for N number of assay plates:

V = [(2 × 10^6) x (N × 5)]/Y

(*where Y is the cell count per ml of the routine cultures*)

Transfer V volume of GenM-C01 and GenM-T01 cell suspensions from the routine cultures to separate sterile centrifuge tubes. Harvest cells by centrifugation at 1400 rpm (~ 340 RCF) for 5 minutes at room temperature. Decant the routine culture medium and resuspend cells by gentle swirling or pipetting in 5 - 10 ml pre-warmed PBS (or D-PBS). Harvest cells a second time at 1400 rpm for 5 minutes, and decant the PBS. Gently resuspend cells in the appropriate volume of complete assay medium to obtain a final density of 2x10^6 cells/ml.

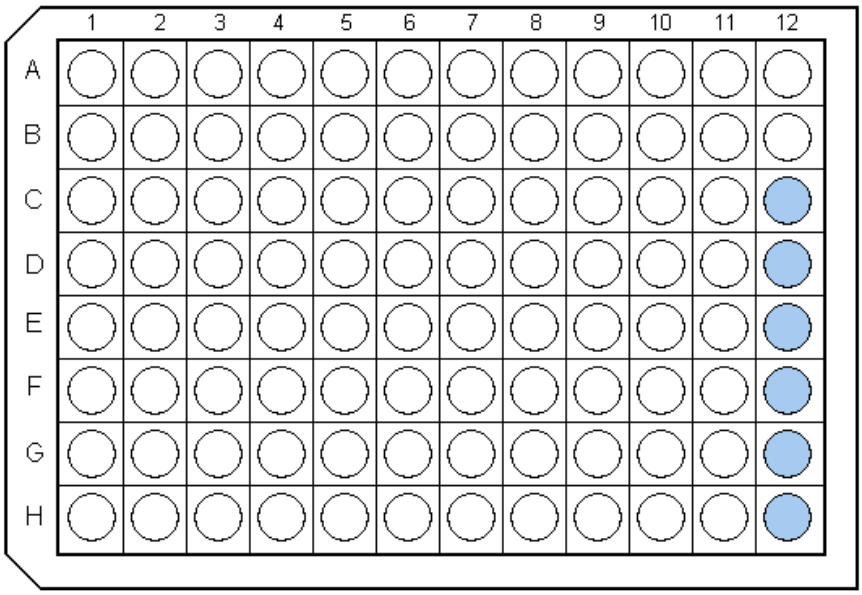
Ensure that cells are fully suspended in media by repeated pipetting or gentle shaking just prior to dispensing.

Microplate set-up Stage 2

1. Dispensing the media contamination controls. [Use a **single-channel** pipette dispensing **75 µl**]

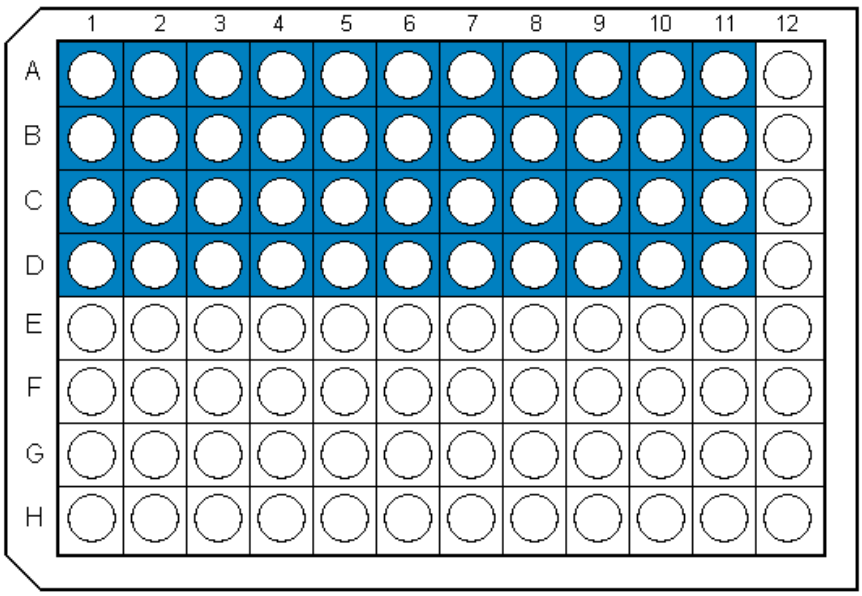
A small volume of **complete assay medium** is used as a standard on the plate in order to demonstrate that the media is clear of contamination. Assay medium for contamination controls should be taken from the same aliquot of complete assay medium used to prepare the cell cultures.

Dispense **75 µl** of Gentronix Complete Assay Medium into wells **C12** to **H12**



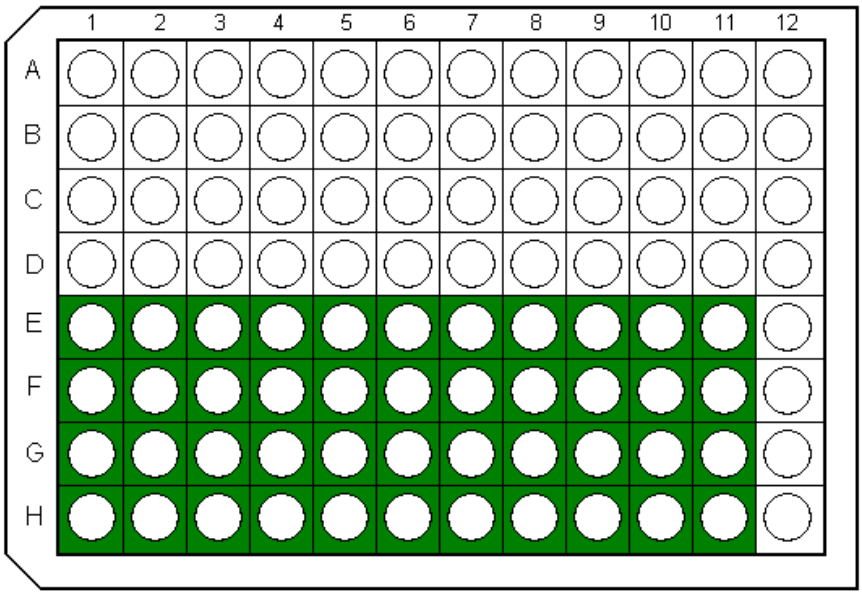
2. Dispensing the GenM-C01 control cell line. [Use an **8 or 12 channel** pipette dispensing **75 µl**]

- Carefully pour the cells / assay medium suspension into a reagent reservoir for dispensing.
- Pipette **75 µl** of **GenM-C01** culture (at 2 × 10 ^{6} cells/ml) into rows **A, B, C** and **D** from column 1 up to and including column 11.

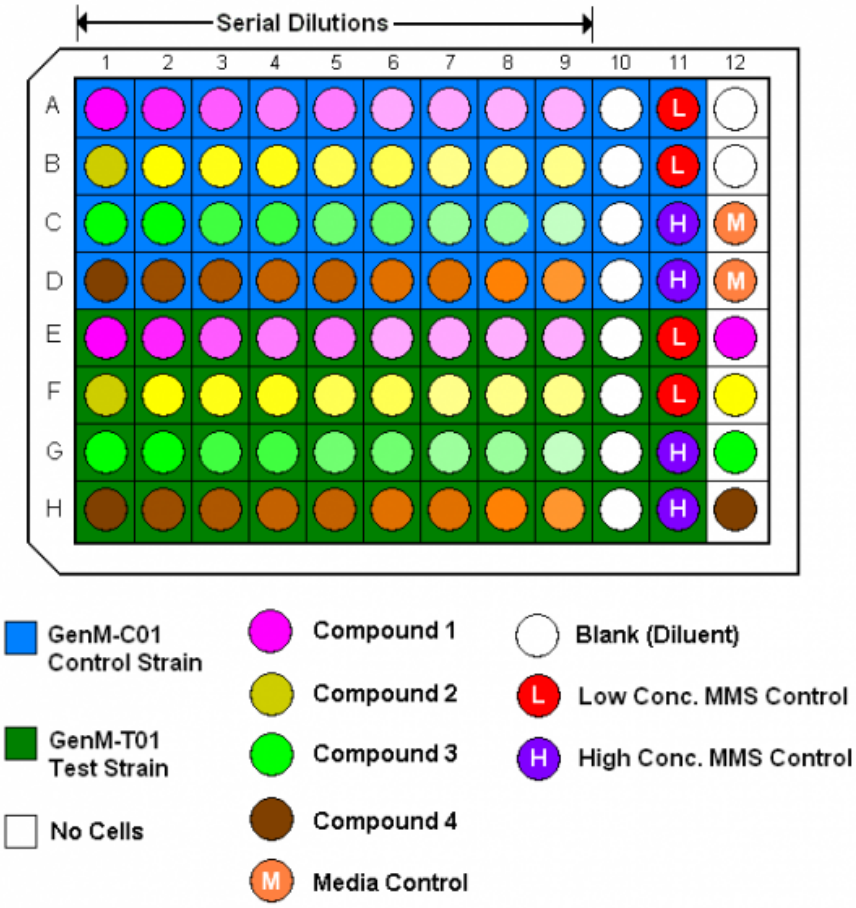


3. Dispensing the GenM-T01 test-strain. [Use an **8 or 12 channel** pipette dispensing **75 µl**]

- Pour the cells / assay medium suspension into a reagent reservoir for dispensing.
- Pipette **75 µl** of **GenM-T01** culture (at 2 × 10 ^{6} cells/ml) into rows **E, F, G** and **H** from column 1 up to and including column 11.



Summary of GSHC Assay Microplate Layout



Covering & Incubation

The plate is now complete. It is recommended that plates are covered with a breathable membrane, being sure to remove both the protective layers, one on either side of the membrane. Label the microplate. Shake the plate gently for 10 to 15 seconds on a microplate shaker (to fully mix the contents of each well) and then incubate at 37 ˚ C, 5% CO_2, 95% humidity, without shaking, for 24 hours. Before analysing in a microplate reader, shake the plate for 10 to 15 seconds on a microplate shaker to thoroughly resuspend the cells and carefully remove the breathable membrane. After reading, recover the microplate with a fresh breathable membrane and incubate for a further 24 hours. After the second period of incubation, again shake the plate for 10 to 15 seconds on a microplate shaker to thoroughly

resuspend the cells and carefully remove the breathable membrane, before taking a final set of microplate readings at the 48 hour time point.

Plates should be incubated for a minimum of 24 hours before the first analysis in the plate reader to allow the cells to undergo a complete division. The second analysis should be made after 48 hours ± 2 hours.

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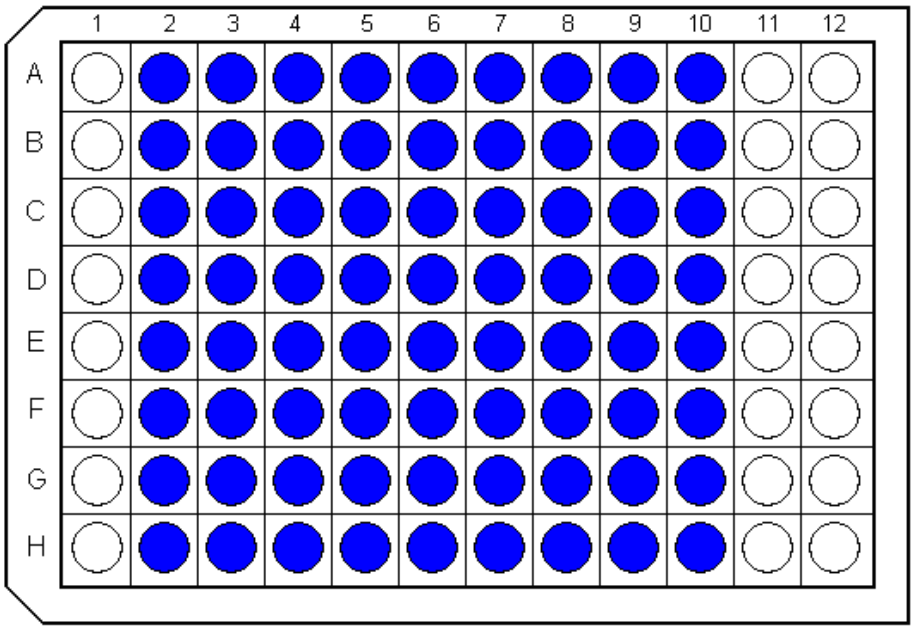
GSHC+S9 Assay:

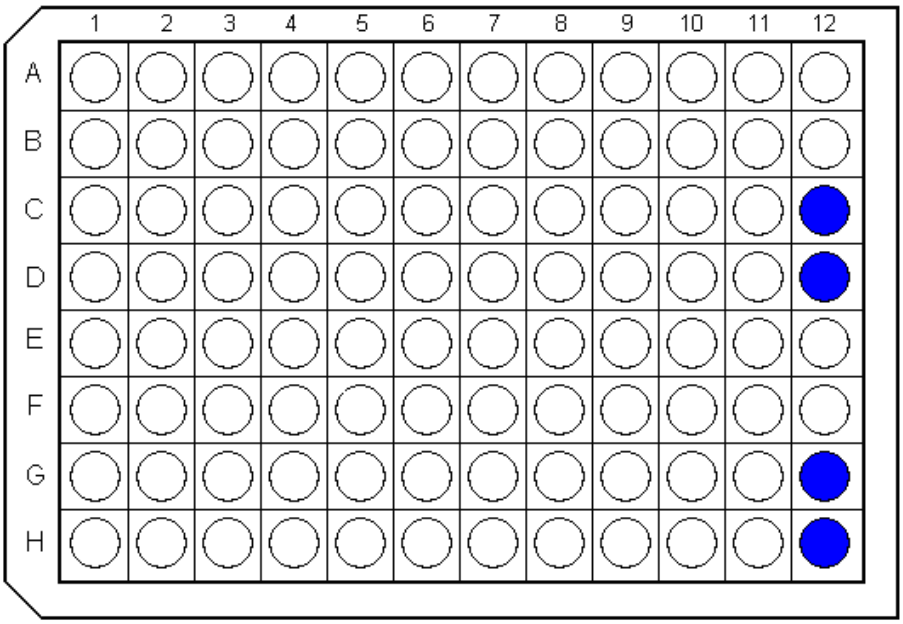
Microplate set-up Stage 1

1. Dispensing the test diluent [Use an **8-channel** pipette dispensing **60 µl**]

- Dispense **60 µl** of diluent into all wells in **columns 2** through to **10**.



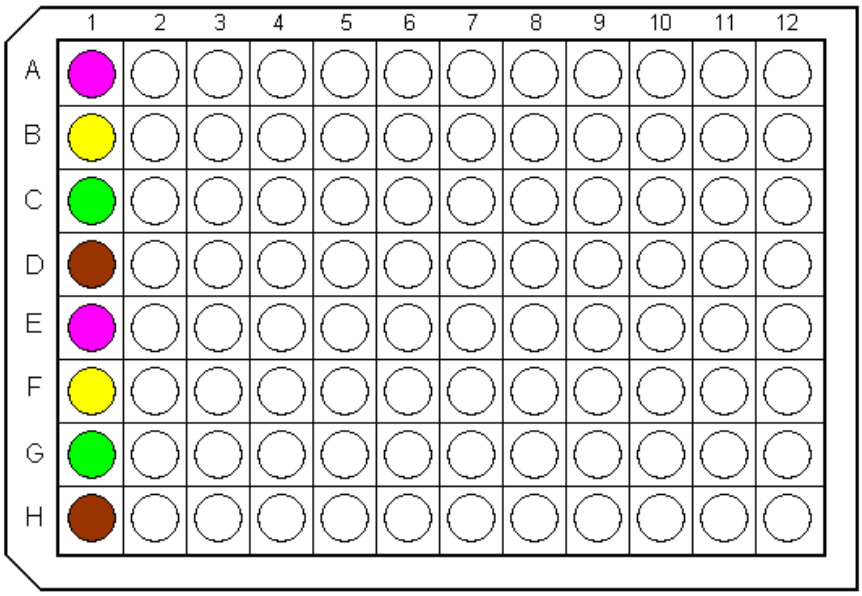
- Dispense **75 µl** diluent into wells **A12, B12, E12** and **F12**.



2. Dispensing the test compounds. [Use a **single-channel** pipette dispensing **120 µl**]

- Dispense **120 µl** of test compound 1 into wells **A1** and **E1**.
- Dispense **120 µl** of test compound 2 into wells **B1** and **F1**.
- Dispense **120 µl** of test compound 3 into wells **C1** and **G1**.

- Dispense **120 µl** of test compound 4 into wells **D1** and **H1**.

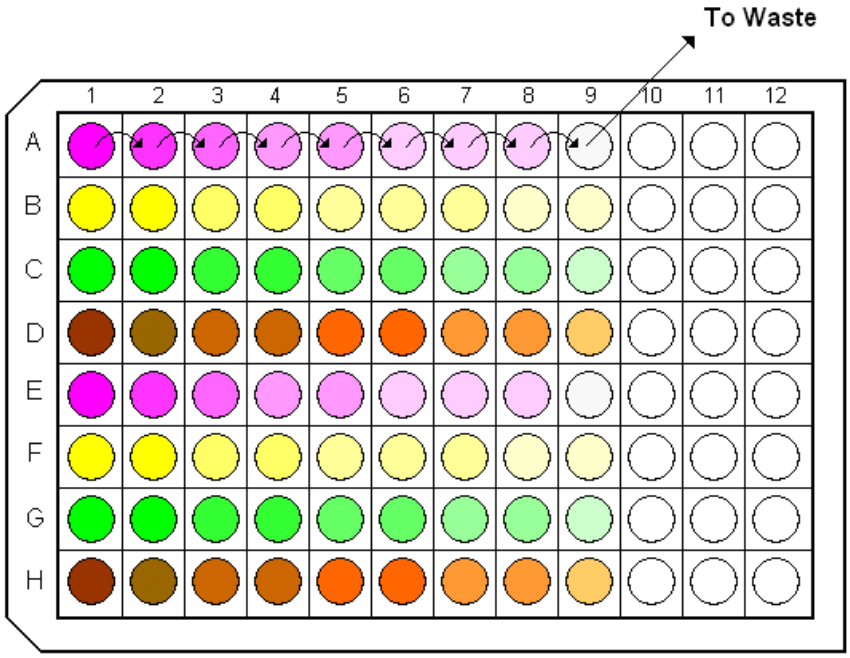


3. Serially diluting the test compounds. [Use an **8-channel** pipette dispensing **60 µl**]

- Aspirate **60 µl** from all wells in column 1 and dispense into column 2.

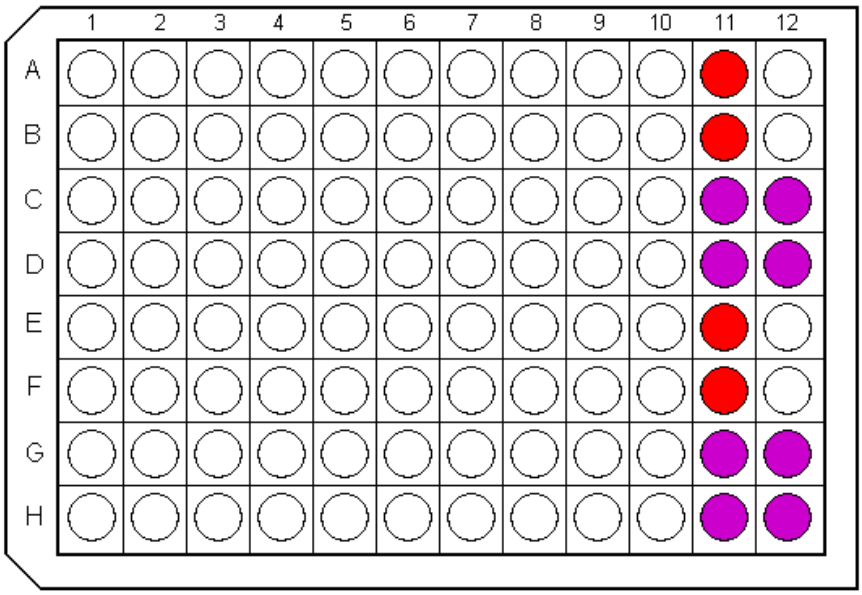
When dispensing into these wells mix the contents by repeated aspiration / dispense, or by using the mix feature common to many electronic auto-pipettes

- Using the same tips, repeat the entire process for the next column to the right, starting with an aspiration of **60 µl** from column 2.
- Repeat this procedure up to and including column 9. After mixing the well contents in column 9, aspirate **60 µl** and discard to waste.



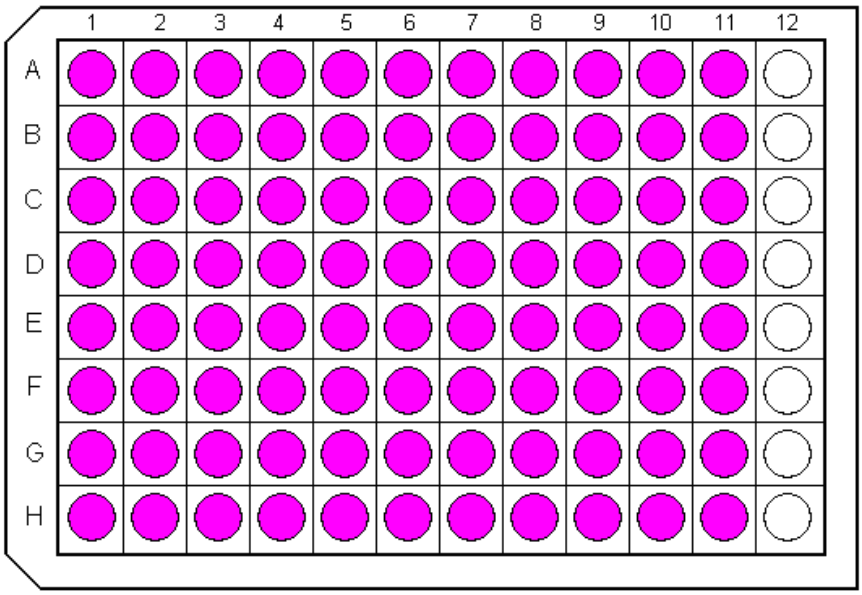
4. Dispensing the standard control compounds. [Use a **single-channel** pipette dispensing **60 µl**]

- Dispense **60 µl** of the **CPA LOW** control into wells **A11, B11, E11** and **F11**.
- Dispense **60 µl** of the **CPA HIGH** control into wells **C11, D11, G11 , H11, C12, D12, G12** and **H12**.

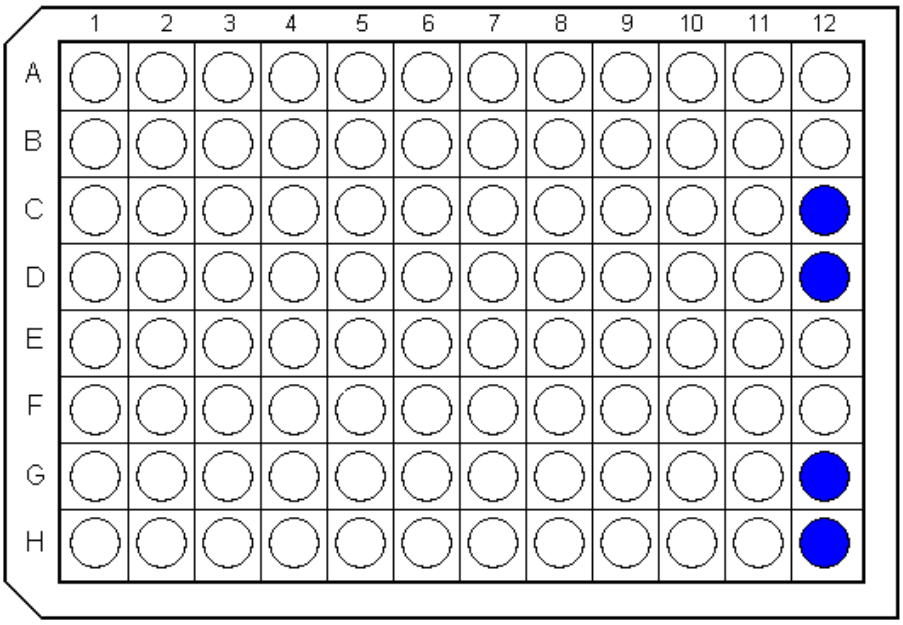


Microplate set-up Stage 2

1. **Combine S9 and Co-factor mix.**
   - Add **200 µl** S9 to 1.8 ml vial of S9 co-factor mix.
   - Once S9 is added to co-factors, the mix should be used **immediately**.
2. **Addition of S9 mix to wells.** [Use a **single-channel** pipette dispensing **15 µl**]
   - Dispense **15 µl** of S9 mix into all wells in columns **1** to **11.**



3. **Addition of diluent to the non-S9 controls.** [Use a **single-channel** pipette dispensing **15 µl**]
   - Dispense **15 µl** of diluent into wells **C12, D12, G12** and **H12.**



Preparation of the cell lines for addition to the microplate

For each assay plate, prepare 5 ml suspensions of GenM-C01 and GenM-T01 cells at a density of 2 × 10^6 cells/ml in exposure medium as follows:

If you have not already done so, calculate the cell density from the routine cultures of GenM-C01 and GenM-T01 cells.

Note: For use in the assay the cell culture densities must be between 5 x 10^5 cells/ml and 1.2 x 10^6 cells/ml. If the cell culture density of either GenM-C01 or GenM-T01 lies outside of this range the assay should not be run and the cells should be passaged for a subsequent day.

A total of 1 × 10^7 cells (i.e. 5 ml at 2 x 10^6 cells per ml) of each cell line are required per assay plate. Use the following equation to calculate the volume (V) of routine cell culture required to prepare cells for N number of assay plates:

$V = [(2 \times 10^6) \times (N \times 5)]/ Y$

(*where Y is the cell count per ml of the routine cultures*)

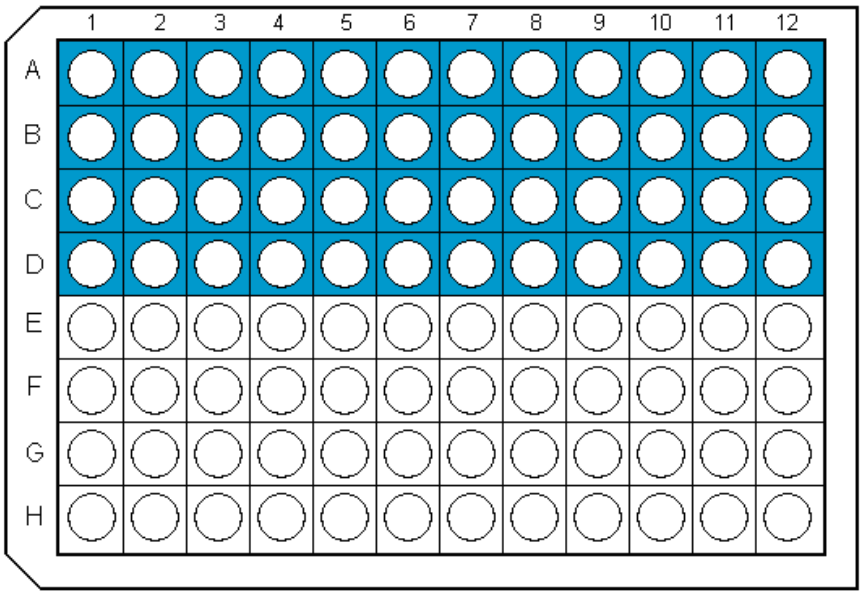
Transfer V (volume) of GenM-C01 and GenM-T01 cell suspensions from the routine cultures to separate sterile centrifuge tubes. Harvest cells at 340 x g for 5 minutes. Decant the routine culture medium and resuspend cells in 5 - 10 ml pre-warmed PBS (or D-PBS). Harvest cells by centrifugation for a second time at 340 x g for 5 minutes, and decant the supernatant. Resuspend cells by gentle swirling or pipetting in the appropriate volume of exposure medium pre-warmed to 37 ° C to obtain final density of 2x10^6 cell/ml.

Ensure that cells are fully suspended in medium by repeated pipetting or gentle shaking just prior to dispensing.

Microplate set-up Stage 3

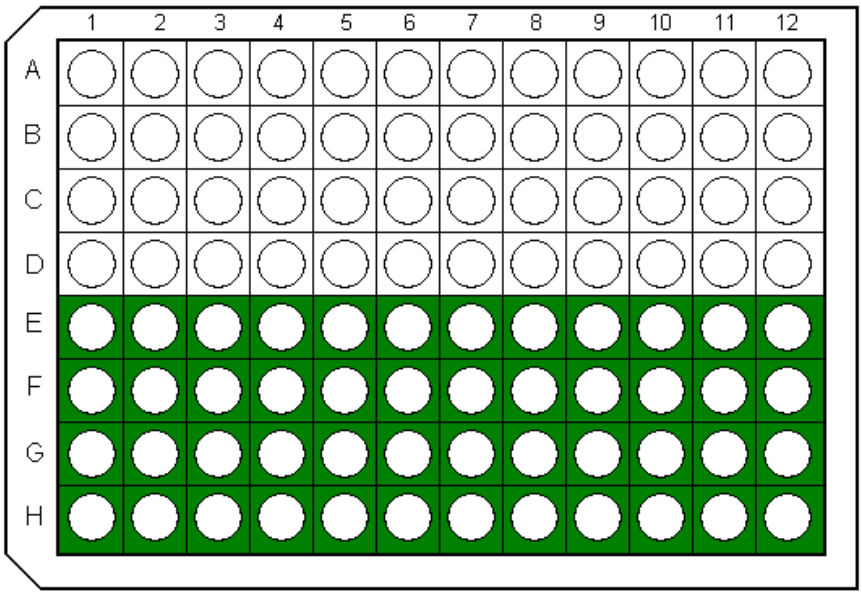
Dispensing the GenM-C01 control cell line. [Use an **8 or 12 channel** pipette dispensing **75 µl**]

- Carefully pour the cells / assay medium suspension into a reagent reservoir for dispensing.
- Pipette **75 µl** of **GenM-C01** culture (at 2 × 10^6 cells/ml) into all wells in rows **A, B, C** and **D**.

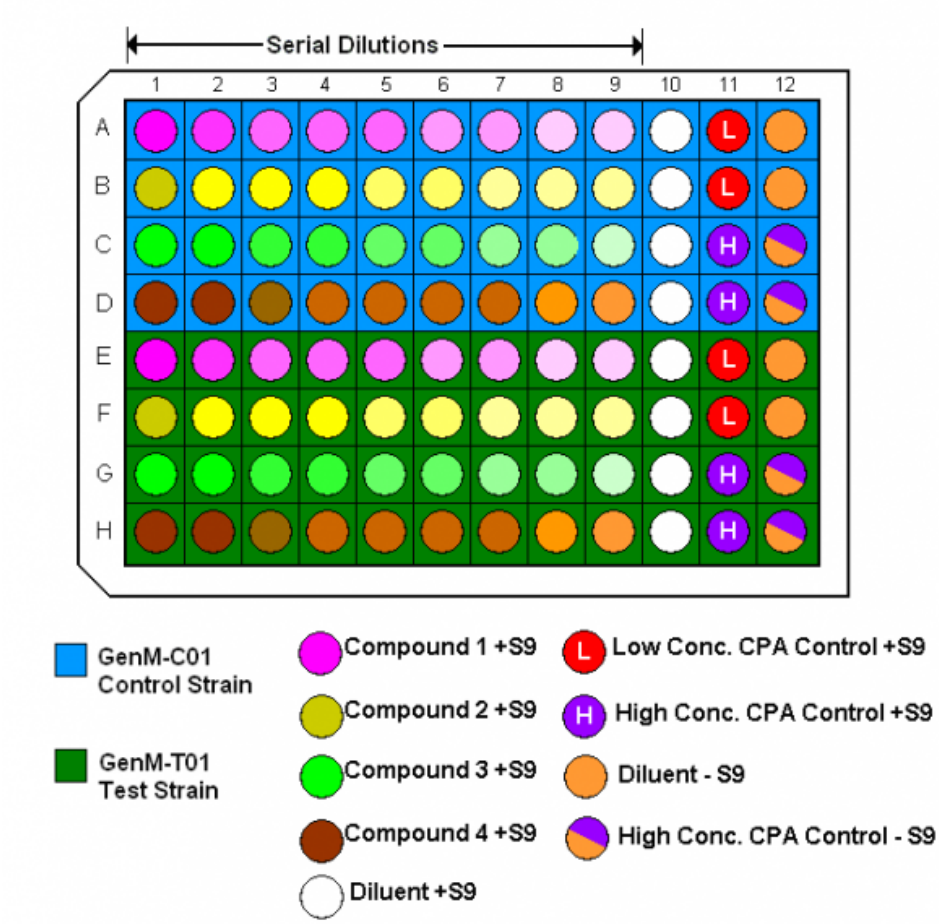


2. Dispensing the GenM-T01 test-strain. [Use an **8 or 12 channel** pipette dispensing **75 µl**]

- Pour the cells / assay medium suspension into a reagent reservoir for dispensing.
- Pipette **75 µl** of **GenM-T01** culture (at 2 × 10^6 cells/ml) into all wells in rows **E, F, G** and **H**.



Summary of GSHC S9 Assay Microplate Layout



Covering & Incubation

The microplate is now complete. It is recommended that plates are covered with a breathable membrane, being sure to remove both the protective layers, one on either side of the membrane. Label the microplate. Shake the plate gently for 10 to 15 seconds on a microplate shaker (to fully mix the contents of each well) and then incubate at 37 ° C, 5% CO_2, 95% humidity, without shaking, for 3 hours.

Wash steps & Recovery period

Following the 3 hr exposure period, washing steps are carried out to remove the S9 mix.

Aspirating and dispensing steps detailed below can be conducted using a [8 or 12 channel pipette] or a correctly configured [liquid handling robotic system/plate washer]. Pipette tips should be changed after aspirating from individual wells to prevent cross-contamination.

- Centrifuge the plate at 340 x g for 5 minutes [requires swing-out microplate rotor].
- Carefully aspirate 120 µl of media from all the wells and discard, taking care not to disturb the pellet.
- Add 120 µl of pre-warmed D-PBS to all the wells, seal with a sterile membrane and resuspend the cells using a microplate shaker.
- Centrifuge the plate at 340 x g for 5 minutes and repeat above steps, such that 2 washes with PBS are performed.
- Carefully aspirate 120 µl of the supernatant from all the wells and discard.
- Add 120 µl pre-warmed recovery medium to all wells.
- Cover with a breathable membrane. Incubate for 45 h at 37 ° C, 5% CO_2, 95% humidity.

Immediately prior to data collection by flow cytometry, gently shake plate on microplate shaker for ~ 20 seconds to resuspend cells.

- To each microplate well add 7.5 µl Propidium Iodide (0.5 mg/ml PI made up in sterile D-PBS) giving a final concentration of 23.8 µg/ml.

- Shake the microplate using an orbital shaker.
- Sample from the microplate on the flow cytometer using the GreenScreen HC/S9 acquisition and analysis flow cytometry template, collecting 10,000 events per well. The GFP signal can be detected through the FL-1 channel and the Propidium Iodide fluorescence through the FL-3 channel.

Endpoint Measurement

GSHC:

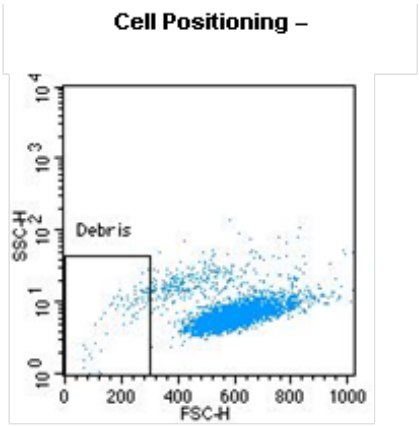
Assays in the absence of metabolic activation employ measurements made at both 24 and 48 hours. Two parameters are collected at each time point using a multi-mode microplate spectrophotometer: optical absorbance at ~ 620 nm (as an estimation of cell numbers and hence proliferation) and reporter GFP fluorescence using FITC wavelengths (485 nm excitation and 535 nm emission).

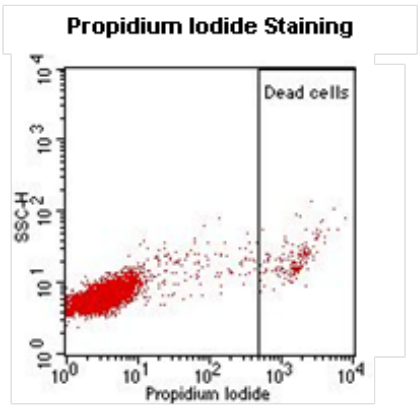
Setting of specific parameters for microplate spectrophotometer data collection is instrument dependent. Most readers deliver 96-well grids of numerical data in either MS Excel ™ format or Excel-compatible format. Data processing software available from Gentronix Ltd is Excel-based and transparent, and currently requires the 96-well grid format of both optical absorbance and fluorescence intensity data.

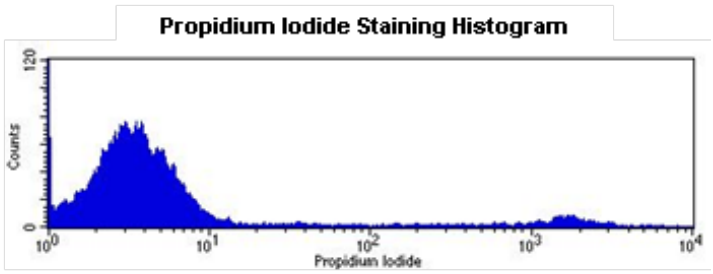
GSHC + S9:

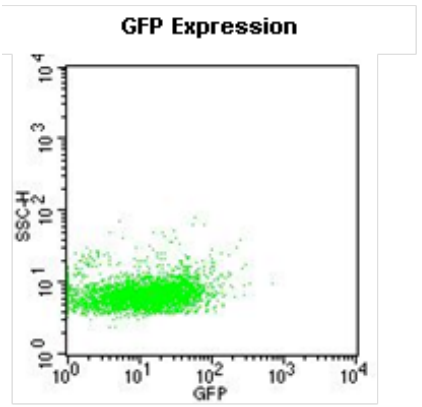
Measurements are only made after 48 hours when S9 metabolic activation is incorporated. Two parameters are collected using a flow cytometer equipped with an air-cooled 488 nm argon – ion laser and a 96-well microplate sampling adaptor, which enables sampling directly from the microplate wells. Green fluorescence from reporter GFP is collected through the 530/30 nm (FL1) band-pass filter whilst red emission from propidium iodide is filtered through the 650LP nm (FL3) filter. Photomultiplier tube voltages are initially set using test cells (expressing GFP) or control cells (not expressing GFP), both stained with propidium iodide. Analysis is restricted to events representing living cells using logical gating relative to PI staining intensity.

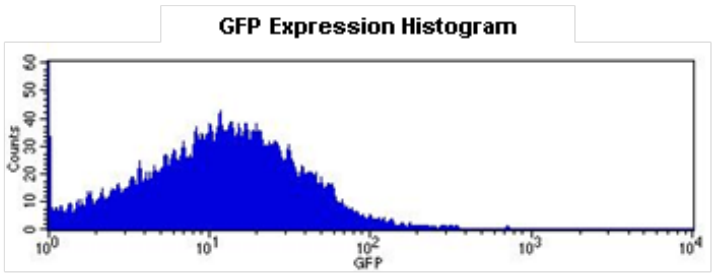
Setting of specific parameters for flow cytometric data collection is instrument and software dependent. However, the following plots and histograms are required for data acquisition and analysis:











Further advice on the set-up of these plots and histograms is available from Gentronix Ltd.

The ‘ Debris ’ and ‘ Dead cell ’ regions created in the cell positioning and propidium iodide staining plots are used in the data acquisition template to exclude events that are not required for the assessment of genotoxicity by flow cytometry in the GSHC S9 assay. The exclusion of events in these regions is achieved using the following logical gating strategy:

Region List:

- R1 = Debris
- R2 = Dead cells.

Gate List:

The following gates should be created using the software gate list manager:

- G1 = NOT “ Debris ” (i.e. NOT R1)
- G2 = NOT “ Debris ” AND NOT “ Dead cells ” (i.e. NOT R1 AND NOT R2).

Gating on plots:

The gating applied to each plot is as follows:

- FSC vs. SSC-H (Plot 1 – Cell positioning) = **No Gating**.
- FL3-H vs. SSC-H (Plot 2 - Cytotoxicity) = **G1: NOT " Debris** " .
- FL1-H vs. SSC-H (Plot 3 – GFP Fluorescence) = **G2: NOT " Debris " AND NOT " Dead cells " .**
- FL3-H Histogram = **G1: NOT " Debris " .**
- FL1-H Histogram = **G2: NOT " Debris " AND NOT " Dead cells " .**

After the successful set-up of the plots, regions and gates, the user is then required to set the data acquisition criteria for the cytometer. GreenScreen HC S9 requires 10,000 events to be collected through the gate **NOT " Debris " AND NOT " Dead cells " .**

Note: The cytometer should be set to collect all events and then to stop collecting events when 10,000 have met the gate of **NOT " Debris " AND NOT " Dead cells " .**

Acceptance Criteria

Test Method Acceptance Criteria

Prior to the provision of GSHC / GSHC+S9 reagents and materials to user laboratories, all kit components undergo a series of quality control assessments at Gentronix. Assay growth medium and components are visually inspected for contamination prior to usage and then spectrophotometrically analysed (absorbance and fluorescence) within performance of the assay for QC purposes. Both methods must be free of contamination in order to pass.

Cryogenically stored batches of cell aliquots are routinely checked for growth in standard cell culture and microscopically for contamination: cell counts must achieve expected levels within a timeframe and cultures must not show microscopic evidence of microbial contamination. Each batch is also mycoplasma tested and must of course be negative. Performance standards must be achieved in performance of the QC GSHC assay with 3 standard compounds and a vehicle control.

Within the user ' s laboratory, cultures of GSHC assay cells (control and test strains – these are the same for GSHC+S9) are passaged for 2 weeks prior to usage in an assay. Within 2-3 days in culture after resuscitation from frozen stocks, cell densities should reach between 2×10^5 and 2×10^6 cells per ml of culture. Cells in culture must be maintained in log phase by passaging every 1 to 4 days. The cell density for both cultures prior to usage of cells in either a GSHC or a GSHC+S9 assay must be between 5×10^5 and 1.2×10^6 cells per ml. Each individual assay microplate requires 1×10^7 cells of each strain (5 ml at a density of 2×10^6 cells per ml).

Cultures are routinely checked for morhphological anomalies. Upon any supicion of contamination, the culture must be destroyed and replaced from fresh stock.

Assay Results Acceptance Criteria

GSHC:

Assay medium sterility – checked using both fluorescence and optical absorbance. The ratio of the absorbance in medium control wells to that in vehicle-only wells must be < 2. The ratio of the fluorescence of the medium control wells to that of the vehicle-only wells must be < 18.

Cytotoxic controls – methyl methanesulfonate (MMS) is used in duplicate at 2 concentrations designated ' high ' and ' low ' . The high MMS standard must reduce the cell density below an 80% threshold (relative to vehicle-treated cells) and it must also produce a cell density lower than that permitted by the low MMS standard. This criterion is an indicator that cells respond properly to the presence of a cytotoxin and that they have grown to an appropriate density for the assay.

Genotoxic controls – MMS is used in duplicate at 2 concentrations designated ' high ' and ' low ' . The high MMS standard must produce a brightness induction ≥ 2 fold, relative to the vehicle-treated controls. The brightness induction from the high MMS standard must also be greater than that from the low MMS standard.

Toxicity limitation – the cell density (relative to the vehicle-treated controls) must not fall below 30% for a compound dose or the data point is invalidated.

Compound Absorbance Check – the ratio of the absorbance in each compound control well (test

compound in vehicle and assay medium) to that in the medium control wells must be < 2. If it is not, the compound may have precipitated or exhibits significant interfering colouration and the user is warned.

Compound Autofluorescence Check – the ratio of the fluorescence from each compound control well to that from the medium control wells must be < 2. If the ratio is > 2, the specific test compound will be annotated as autofluorescent; > 5 and the warning is up-graded to highly autofluorescent, with a suggestion to make a fluorescence polarization measurement.

GSHC+S9:

Population size – mean GFP fluorescence must be derived from 10,000 events (after the exclusion of events classed as debris and dead cells) otherwise the data point is invalidated. A population size of < 10,000 events is caused either by very high toxicity or a flow cytometer problem.

Toxicity limitation – cell death is determined by the intensity and extent of propidium iodide fluorescence which is converted to a relative population survival measure when compared to vehicle-treated controls. If the relative population survival falls below 20%, the data point is invalidated.

Genotoxic controls – cyclophosphamide (CPA) is used at 2 concentrations designated ‘ high ’ and ‘ low. The high CPA standard must produce a GFP fluorescence induction of > 1.3, which must also be higher than that produced by the low CPA standard. If either of these conditions is not met then the assay is invalidated.

Solvent Treated Control Check – at least 2 of 4 vehicle-treated control wells for each cell strain must meet the criterion of 10,000 events (after the exclusion of debris and dead cells) for the assessment of GFP fluorescence. If 3 or more wells do meet this criterion the assay is invalidated.

Metabolic Activation Genotoxic Control – compares the GFP induction for high CPA standard treated cells in the presence and absence of S9. In the presence of S9, a GFP induction of > 1.3 must be produced and this must be higher than that for the same in the absence of S9.

Data Analysis

GSHC:

The fluorescence and absorbance data from the 24 and 48 h incubation are transferred by copy/paste into a Microsoft Excel spreadsheet (see Annex 1 for an example) and automatically converted to graphical dose-response format. Data processing requires only simple arithmetical manipulations. Absorbance data are used to give an indication of the reduction in proliferative potential or relative suspension growth, and these data are normalized to the vehicle-treated control (=100% growth). Fluorescence data are divided by absorbance data to give ‘brightness units’ , the measure of the average GFP induction per cell. These data are normalized to the vehicle-treated control (=1). In order to correct for induced cellular autofluorescence and intrinsic compound fluorescence, the brightness values for the GenM-C01 cell line are subtracted from those of GenM-T01 cells. This makes visual assessment of the data more reliable.

Two thresholds were set from the absorbance data. The first is the threshold at which there is a statistically significant reduction in the proliferative potential or relative suspension growth (RSG). This threshold is not used in data handling but is provided to give an indication to the user that the compound is causing some growth inhibition. It is set at 80% of the maximum extent of cell proliferation on each microplate (i.e. the cell density reached by the vehicle-treated control cells). This is greater than three times the standard deviation in the absorbance of vehicle-treated wells. Mortality is not measured in this assay: 80% RSG does not mean 20% of the cells are dead. A compound is recorded as having an effect on cell proliferation if one compound dilution produces a final cell density below the 80% threshold after 48 h, and ‘-’ is recorded when no compound dilutions produce a final cell density lower than the 80% threshold. The lowest effective concentration (LEC) for growth inhibition is the lowest test compound concentration that produces a final cell density below the 80% threshold. The second threshold is set at 30% RSG. This is a rejection threshold for genotoxicity data and reflects two properties of the system. Firstly, this threshold recognizes the limits imposed by instrumentation: at cell densities lower than 30% RSG, interference in the optical measurements becomes significant due to variation in the background reflectance and absorbance of the microplate. Secondly, this level of growth means that the population of cells in the culture has been unable to complete even one doubling and as such is a toxicity limitation (the normal cell doubling time of the reporter and control cell lines is 20 – 24 h). A breakdown in cell integrity can lead to non-specific DNA damage, although if a cell is dead or dying this is clearly of little genetic consequence: apoptosis in mammalian cells is itself a deliberate manifestation of this.

The genotoxic threshold reflects a statistically significant increase in brightness compared with the vehicle-treated control. It is set at 1.5 (i.e. a 50% increase) and this is greater than three times the standard deviation of the background brightness. A positive genotoxicity result is concluded if one compound dilution produces a relative GFP induction greater than the 1.5 threshold. A negative genotoxicity result is concluded where no compound dilutions produce a relative GFP induction greater than the 1.5 threshold. If a positive genotoxicity result was observed after 24 and/or 48 h the compound was concluded positive for genotoxicity. The LEC for a positive genotoxicity result is the lowest test compound concentration that produces a relative GFP induction greater than the 1.5 threshold.

GSHC+S9:

A data acceptance criterion was set as follows: 10,000 gated (viable) events per sample had to be collected in order for a sample to be included in the genotoxicity assessment. The number was constrained by several factors. Logical gates were applied as follows to restrict data analysis to living cells. A rectangular region was created in the bivariate forward scatter channel (FSC) versus side scatter channel (SSC) plot encompassing the non-cellular debris (see dot plots in Figure 1 below). A NOT gate was assigned to remove the debris and this gate was applied to all the bivariate SSC versus fluorescence plots. A further region was created in the SSC versus PI bivariate plot to allow the exclusion of dead cells from the GFP analysis. This region was predetermined using formalin-fixed cells which had been exposed to 25 µ g/ml PI. A second NOT gate was assigned to remove dead cells and this was applied to the bivariate SSC versus GFP fluorescence plot. Viable events were determined by the collection of 10,000 events that fulfilled the criteria determined by the ‘ NOT debris and NOT dead cells ’ gate, whilst the total number of cellular events was determined by the ‘ NOT debris ’ gate. Toxicity and relative population survival were calculated as percentages using the following equations:

Level of toxicity = (1 – (Number of Viable Events / Total Number Cellular Events)) x 100;

Rel. pop. survival = ((100 x Level of toxicity for compound dose) / (100 x Level of toxicity for vehicle - treated control)) x 100.

Relative population survival provides a measure of the survival of treated cells relative to the survival levels in the vehicle-treated control. GFP induction (Fi) was calculated using the mean channel fluorescence intensity (MCFI) from treated test cells corrected for background fluorescence, relative to that of the background corrected vehicle-treated test cells. Background fluorescence was removed by subtraction of the control strain fluorescence (FC) from the reporter strain fluorescence (FT), thus allowing the resolution of GFP through any interfering green autofluorescence arising from cellular constituents or test article. The following equation was used to calculate GFP induction:

Fi = (Treated FT -Treated FC) / (Vehicle-treated FT-Vehicle treated FC)

To simplify data processing from a 96-well microplate, a simple arithmetic programme was developed in MS Excel, analogous to that used in GSHC (see Annex 2 for an example) and automatically converted to graphical dose-response format. This generates graphical dose – response curves from the brightness data (genotoxicity) and cell viability, as well as automatic notification of a positive result, with the associated LEC. A cytotoxicity threshold is set at a decrease in population survival to below 90%, i.e. an increase in cytotoxicity above 10% in the treated population relative to the vehicle treated control. The cytotoxicity result is based on the percentage of cells which are permeable to the nucleic acid stain PI, relative to the vehicle treated control cellular population.

In the GSHC, the significance threshold for positive genotoxicity results is defined as an increase of equal to or greater than three times the standard deviation (SD) of the mean fluorescence intensity in vehicle-treated cells. This translates to a decision threshold of 1.5-fold induction. In the same way in GSHC S9, flow cytometry data from vehicle and non-genotoxin-treated cells were used to define a level of GFP induction that is three times the SD and accordingly, the statistically significant increase in GFP induction is set at 1.3-fold. Acceptance of assay data is also dependent upon the degree of toxicity caused by higher concentrations of the test article. The toxicity limit of 80% (minimum relative population survival of 20% compared to the vehicle-treated control, assessed using PI fluorescence) was selected.

Figure 1 extracted from Jagger & Tate *et al*., 2009:

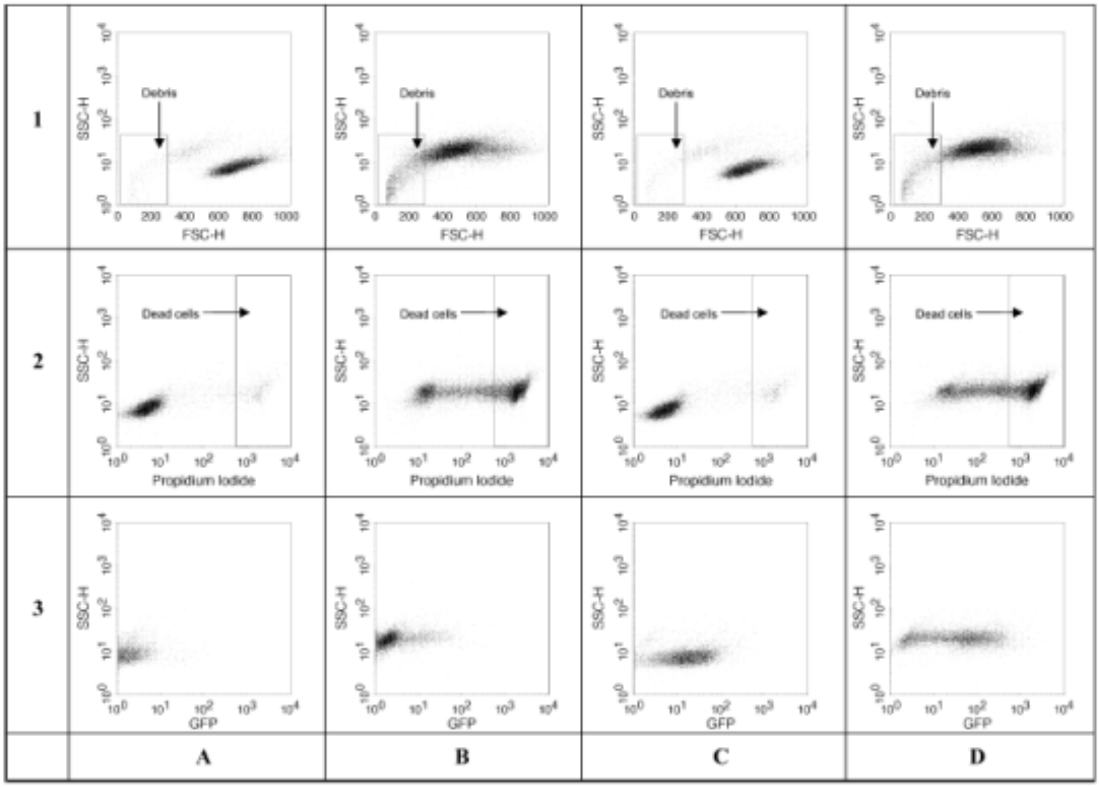


Fig. 1. FCM detection of the *GADD45a–GFP* reporter. For an event to be included in the final fluorescence measurement, it must first fulfil specific gating criteria (see text). Panels (A-1–3) contain resting control cells. Panels (C-1–3) contain resting reporter cells. Panels (A-1) and (C-1) show the morphological placement of the cells allowing removal of non-cellular debris. Panes (A-2) and (C-2) demonstrate the number of events staining with PI allowing the subsequent removal of these dead cells. Panels (A-3) and (C-3) house the cells which are expressing GFP fluorescence. Panels (B-1–3) and (D-1–3) contain control and reporter cells, respectively, following a 48-h exposure to 0.1 μg/ml camptothecin.

Prediction Model

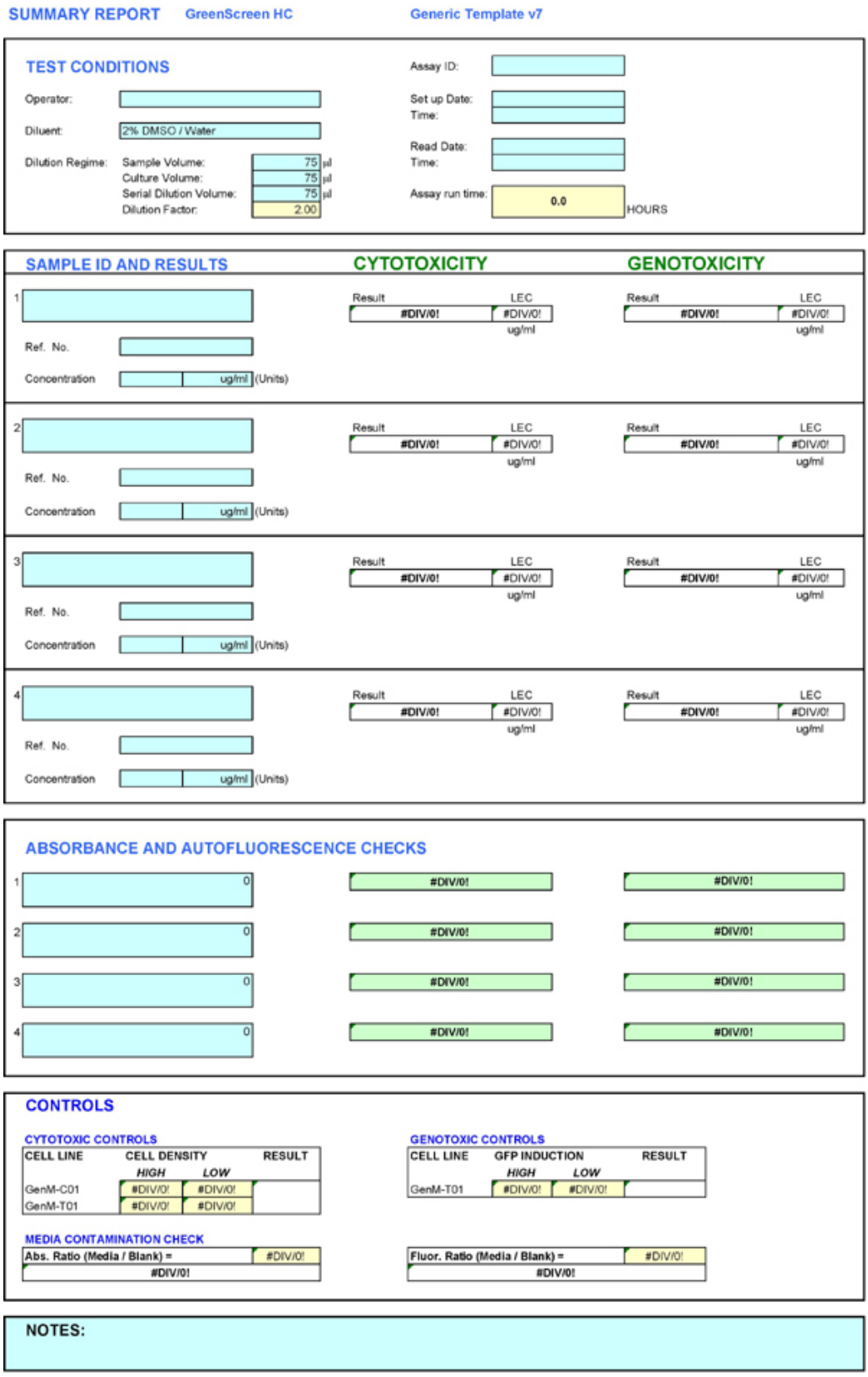
Published GSHC data demonstrate its high specificity and high sensitivity, together providing high overall accuracy in the prediction of in vivo genotoxicity results and genotoxic carcinogenicity. Currently used *in vitro* mammalian genotoxicity assays are sensitive for carcinogens (> 70%) but have poor specificity (in vivo testing for resolution).

Using the analysis models and acceptance criteria set out above, the GreenScreen HC assay gives a relevant indication of the genotoxic potential of a test compound. For a set of 155 compounds, for which data from other genotoxicity assays (*in vitro* and *in vivo*) and carcinogenicity studies are available, the GADD45a-GFP assay was 85% sensitive and 92% specific for *in vivo* genotoxins. These figures were 88% and 95% for the prediction of genotoxic carcinogens.

Annexes

Annex 1 – GSHC data processing

Summary report page (blank):



SUMMARY REPORT GreenScreen HC Generic Template v7

TEST CONDITIONS

Operator:

Diluent: 2% DMSO / Water

Dilution Regime: Sample Volume: 75 µl; Culture Volume: 75 µl; Serial Dilution Volume: 75 µl; Dilution Factor: 2.00

Assay ID:

Set up Date: Time:

Read Date: Time:

Assay run time: 0.0 HOURS

SAMPLE ID AND RESULTS	CYTOTOXICITY		GENOTOXICITY	
	Result	LEC	Result	LEC
1	#DIV/0!	#DIV/0! ug/ml	#DIV/0!	#DIV/0! ug/ml
Ref. No.				
Concentration	ug/ml (Units)			
2	#DIV/0!	#DIV/0! ug/ml	#DIV/0!	#DIV/0! ug/ml
Ref. No.				
Concentration	ug/ml (Units)			
3	#DIV/0!	#DIV/0! ug/ml	#DIV/0!	#DIV/0! ug/ml
Ref. No.				
Concentration	ug/ml (Units)			
4	#DIV/0!	#DIV/0! ug/ml	#DIV/0!	#DIV/0! ug/ml
Ref. No.				
Concentration	ug/ml (Units)			

ABSORBANCE AND AUTOFLUORESCENCE CHECKS

1	0	#DIV/0!	#DIV/0!
2	0	#DIV/0!	#DIV/0!
3	0	#DIV/0!	#DIV/0!
4	0	#DIV/0!	#DIV/0!

CONTROLS

CYTOTOXIC CONTROLS

CELL LINE	CELL DENSITY		RESULT
	HIGH	LOW	
GenM-C01	#DIV/0!	#DIV/0!	
GenM-T01	#DIV/0!	#DIV/0!	

GENOTOXIC CONTROLS

CELL LINE	GFP INDUCTION		RESULT
	HIGH	LOW	
GenM-T01	#DIV/0!	#DIV/0!	

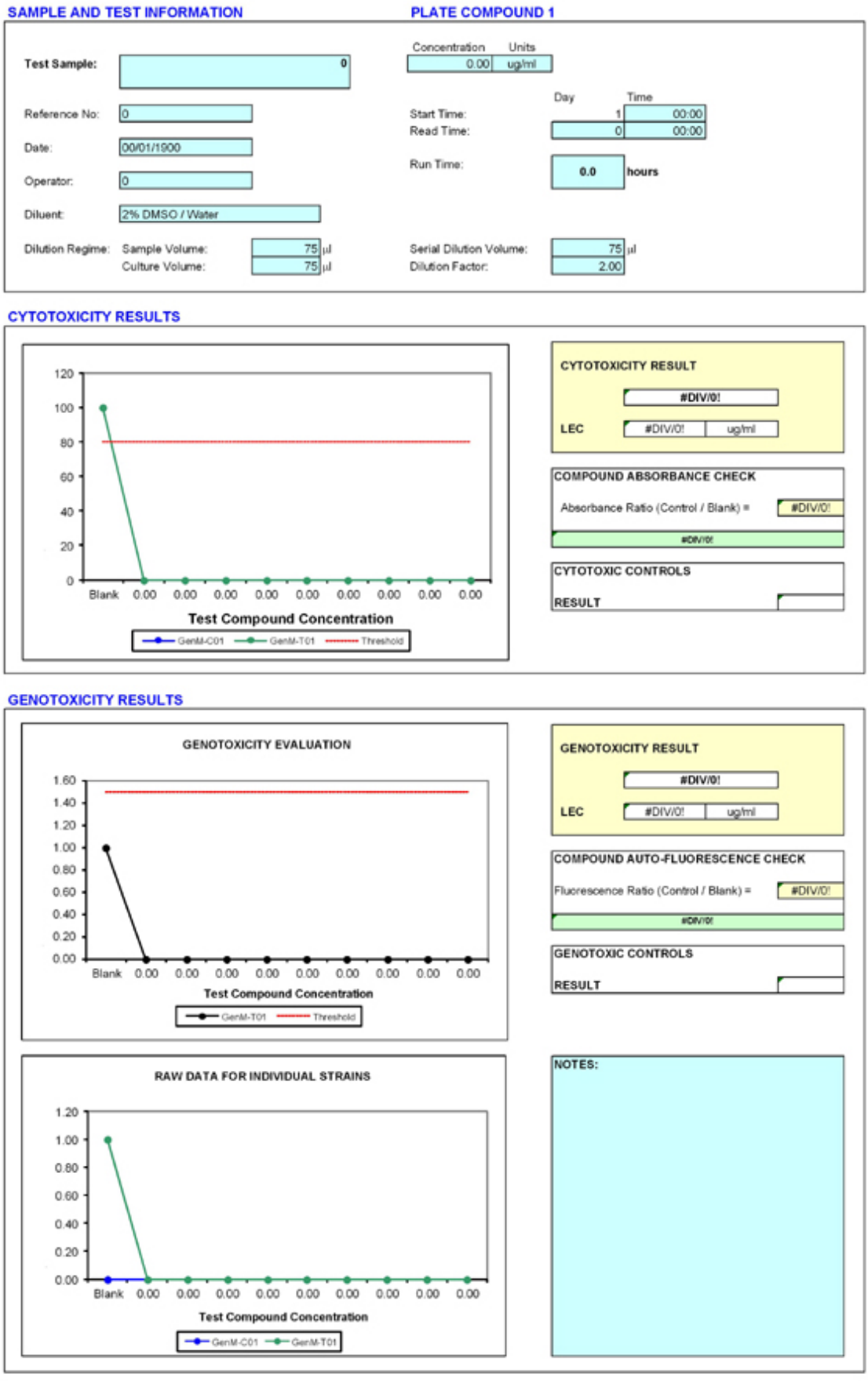
MEDIA CONTAMINATION CHECK

Abs. Ratio (Media / Blank) =	#DIV/0!
#DIV/0!	

Fluor. Ratio (Media / Blank) =	#DIV/0!
#DIV/0!	

NOTES:

Example compound report page (blank):



Raw data input page (blank):

Raw Absorbance Data

<>	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

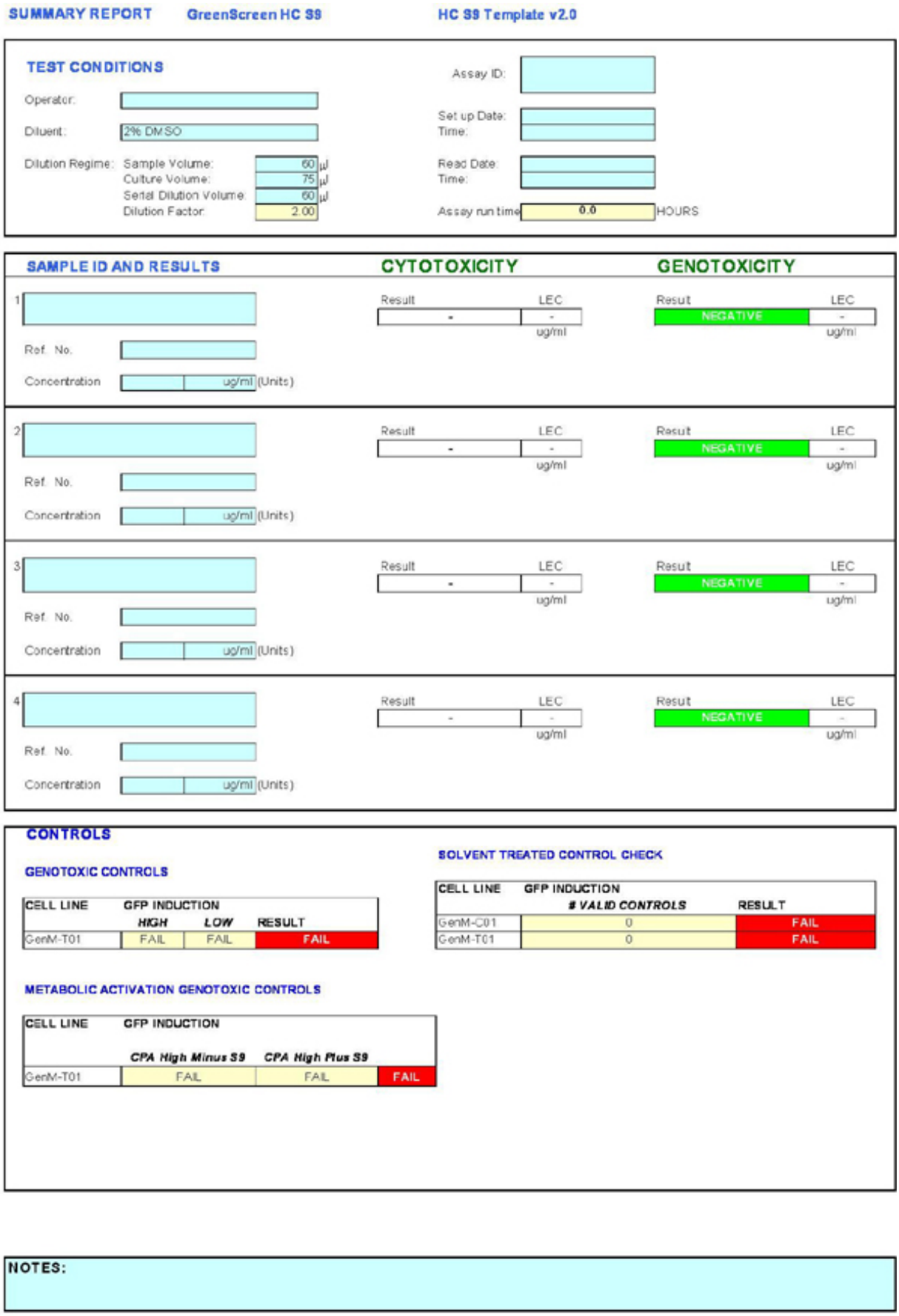
Raw Fluorescence Data

<>	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Microplate Reader Settings and Notes

Annex 2 – GSHC+S9 data processing

Summary report page (blank):



SUMMARY REPORT GreenScreen HC S9 HC S9 Template v2.0

TEST CONDITIONS

Operator:
Diluent: 2% DMSO
Dilution Regime: Sample Volume: 60 µl; Culture Volume: 75 µl; Serial Dilution Volume: 60 µl; Dilution Factor: 2.00
Assay ID:
Set up Date: Time:
Read Date: Time:
Assay run time: 0.0 HOURS

SAMPLE ID AND RESULTS	CYTOTOXICITY Result	CYTOTOXICITY LEC	GENOTOXICITY Result	GENOTOXICITY LEC
1	-	- ug/ml	NEGATIVE	- ug/ml
2	-	- ug/ml	NEGATIVE	- ug/ml
3	-	- ug/ml	NEGATIVE	- ug/ml
4	-	- ug/ml	NEGATIVE	- ug/ml

Ref. No.
Concentration ug/ml (Units)

CONTROLS

GENOTOXIC CONTROLS

CELL LINE	GFP INDUCTION HIGH	GFP INDUCTION LOW	RESULT
GenM-T01	FAIL	FAIL	FAIL

SOLVENT TREATED CONTROL CHECK

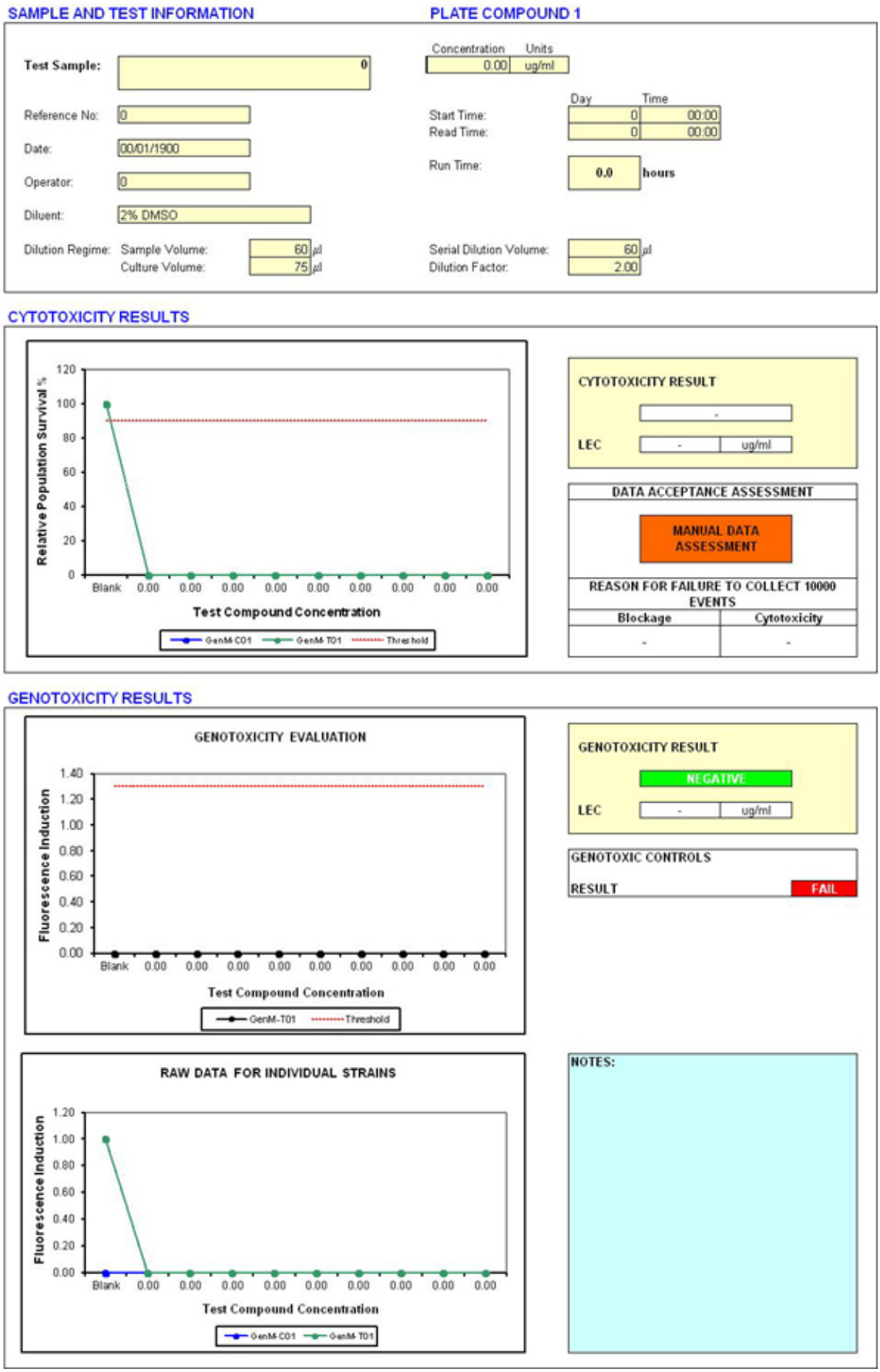
CELL LINE	GFP INDUCTION # VALID CONTROLS	RESULT
GenM-C01	0	FAIL
GenM-T01	0	FAIL

METABOLIC ACTIVATION GENOTOXIC CONTROLS

CELL LINE	GFP INDUCTION CPA High Minus S9	CPA High Plus S9	
GenM-T01	FAIL	FAIL	FAIL

NOTES:

Example compound report page (blank):



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