CHAPTER 13:
THE APPLICABLE USE OF THE HPRT GENE MUTATION ASSAY AS A PRACTICAL TOOL IN MUTAGENESIS AND DNA REPAIR STUDIES

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SUMMARY

The HPRT gene mutation assay is a notable tool that detects for genotoxic substances and allows for the isolation and screening for inducible mutation types. As with the thymidine kinase (TK) mouse lymphoma assay (MLA), the HPRT gene mutation assay is considered a significant tool as set out in the guidelines for mammalian gene mutation tests (OECD 1997). Since its refinement the HPRT assay has been widely utilised in mechanistic studies using human knock-out cell lines for DNA repair, providing details of the mode of action (MOA) of the test substance. This chapter provides up-to-date methodology for carrying out the assay in different cell lines in the presence and absence of metabolism with relevant technical information and emerging advances in statistical analysis of data generated from the HPRT gene mutation assay.

Key words: HPRT, Mutagenicity, DNA repair, human lymphoblastoid cells

1. RECOMMENDED USE OF THE HPRT ASSAY

There are three main features of the mammalian *HPRT* gene mutation assay which have led to its wide use;

a) The target gene is encoded on the mammalian X-chromosome and consequently it is easy to select for loss of function mutants in cells derived from males, which in mammals are heterogametic for sex chromosomes.

b) The biochemical selection systems for loss of function with cells that survive in presence of 6-thioguanine (6-TG) and/or 8-azoguanine, are simple and effective (Caskey and Kruh 1979).

c) Also an advantage of the *HPRT* gene is that mutations in the same gene can be compared between cell lines, experimental animals and with humans (Chen et al. 2002).

Human lymphoblastoid cell lines (Furth et al. 1981) were first used for *HPRT* assays. Chinese hamster ovary, CHO cells then became the main cell line (Moore et al. 1991) and these adherent cells have been the most widely used cells to date. However, the test is relatively flexible and the human lymphoblastoid AHH-1 and MCL-5 suspension cell lines can also be used (Crespi and Thilly 1984; Crespi et al. 1991). The AHH-1 cell line is very suitable for these experiments, and because it is heterozygous at the $TK^{+/-}$ locus we were able to validate our *HPRT* results by carrying out both the HPRT and TK gene mutation assays alongside one another. *In vivo* experiments can also be performed using this methodology, and mice or rats are commonly treated with the test substance by an appropriate route of exposure. After a fixation period, the rodent lymphocytes are removed and established.

T-lymphocytes are particularly useful to examine because they circulate throughout many tissues, which allows them a greater chance to come into contact with an administered mutagen than cells that are permanently resident in a single tissue. T-lymphocytes are also long-lived in circulation and they continue to undergo cell division, which makes the identification of mutant cells possible. In addition to T-lymphocytes, mutant frequency has also been determined in many cells, including those from the spleen, kidney, thymus and lymph nodes.

2. MOLECULAR PRINCIPLES OF THE *HPRT* ASSAY

HPRT is a key enzyme in the purine salvage pathway with more than 300 disease associated mutations reported (Torres and Puig 2007; Jinnah et al. 2010). The main being through partial enzyme deficiency leading to gouty arthritis and complete deficient leading to Lesch-Nyhan disease (Jolly 1986).

The *HPRT* gene is located on the X-chromosome and encodes for hypoxanthine-guanine phosphoribosyl transferase (HPRT) protein, which plays a key role in the purine salvage pathway. HPRT catalyses the transformation of purines (hypoxanthine, guanine, or 6-mercaptopurine) to monophosphates, which are therefore cytotoxic to HPRT proficient cells. Cells with mutations in the *HPRT* gene have lost the HPRT enzyme and thus survive treatment with purine analogues.

As large losses in the X-chromosome lead to cell lethality, only small changes, such as point mutations and exon deletions are detected in the *HPRT* gene. The spontaneous MF is also lower at the *HPRT* locus, than the *TK* locus, as high proportions of mutations at *HPRT* are lethal (but not at *TK*). These events include non-disjunction and translocation which are known to lead to viable *TK* mutants but not viable *HPRT* mutants. After a chemical insult, further multiple events are required to transform DNA changes (pro-mutagenic DNA lesions) into selectable phenotypes:

a) Fixation of the mutation.
b) Reduction of the pre-existing enzyme to a level with no biological activity.

Fixation of a mutation requires the initial lesion in the DNA (i.e. adduct, strand break or damage to DNA dependent proteins) being translated into a DNA sequence change, such as point mutation, deletion or loss. For a point mutation, the mutant strand must be separated from the wild type strand by cell division, thus one of the progeny cells no longer produces active mRNA and/or protein. Point mutations can only occur after the cells have undergone cell division, as the lesion affecting the base may be removed or the base may be repaired by DNA repair. The mutation is therefore only relevant in these assays once it has been incorporated into both strands. For selection, the existing enzyme or mRNA must be reduced either by cell division or degradation to non-functional levels, so that the original phenotype can no longer be identified.

Cells are incubated in microwell culture plates with the selective agent 6-thioguanine, a purine analogue that is a substrate for HPRT and is toxic to non-mutant cells. A significant increase in mutant frequency in treatment cultures compared with controls indicates the test chemical has induced mutation at the *HPRT* locus. The average spontaneous mutant frequency at the *HPRT* locus is in the range of 10^{-6} (Johnson 2012). Using standard techniques, further molecular analysis of *HPRT* mutations can be performed, if desired. Mutation spectra utilising HPRT mutants from AHH-1 cells treated with the direct-acting genotoxicant methyl-nitrosourea (MNU) (Thomas et al. 2013) and *HPRT* from harvested liver cells from transgenic mice treated with phenobarbital (Shane et al. 2000) have provided invaluable insight into mutation hotpots, mutation type and frequency.

3. MATERIALS

3.1. Cell Lines:

Our group in Swansea University has recently used the AHH-1 cell line to investigate the dose response relationships of DNA reactive genotoxic agents in the low dose region of exposure (Parry et al. 2005; Doak 2008). The AHH-1 cell line was very suitable for these experiments, and because it is heterozygous at the $TK^{+/-}$ locus we were able to validate our *HPRT* results by carrying out both the HPRT and TK gene mutation assays alongside one another.

Other suitable cell lines for the *HPRT* assay include L5178Y mouse lymphoma cells, the CHO, AS52 and V79 lines from Chinese hamsters (Tindall et al. 1986; Tindall and Stankowski Jr 1989; OECD 1997), and AHH-1, MCL-5 and TK6 human lymphoblastoid cells (Crespi and Thilly 1984; Moore et al. 1987; Crespi et al. 1991; Parry et al. 2005). The *HPRT* locus is on the X-chromosome and therefore primary male cell lines can also be used to study mutagenic effects in mice and rats (van Dam et al. 1992; Tates et al. 1994) and this methodology can also be used for human bio-monitoring (Albertini 2001; Parry et al. 2005). In this chapter we will focus on the *in vitro HPRT* assay mainly in human lymphoblastoid cell lines.

Each cell line requires specific culture medium and this along with the cell culture conditions are stated in the batch details, provided upon purchase of the cell line.

- L5178Y mouse lymphoma cells and Chinese Hamster Ovary (CHO) cells, have a published spontaneous MF of 2-50x10^6 (DeMarini et al. 1988).
- AHH-1 and MCL-5 human lymphoblastoid cells have a published spontaneous MF of 6-80x10^6 (Gentest 1994; Doak et al. 2007).

3.2. Compounds Required For The *HPRT* Gene Mutation Assay:

HAT (Hypoxanthine Aminopterin Thymidine) supplement is added to culture medium at the mutant cleansing stage. The aminopterin in HAT medium blocks the salvage pathway, leaving cells reliant on the endogenous pathway i.e. *HPRT* and *TK*, and therefore *HPRT* $^-$ and *TK* $^{-/-}$ mutants are killed. This reduces the spontaneous (background) MF values and is a crucial step. Concentrated HAT medium may be purchased from Sigma or GIBCO and diluted in sterile water. Alternatively 1 x HAT media may be made thus: 100µM hypoxanthine, 1 µM aminopterin, 20 µM thymidine. For freezer stock it is recommended to freeze aminopterin separately thus: dissolve 136 mg hypoxanthine and 48.4mg thymidine in a total volume of 100ml sterile water to give 100X stock. Filter sterilise and freeze 1 ml aliquots. Protect from light. Dissolve 4.4 mg aminopterin in a few ml of sterile 0.1N NaOH up to 100ml sterile water to give 100X stock. Ensure pH is 7.0, adjust with HCl if above 7.0. filter sterilise and store in 1 ml aliquots at -80^{o}C. Protect from light.

Following HAT treatment, HT (Hypoxanthine Thymidine) supplement is added to culture medium and both the *de novo* nucleotide biosynthesis pathway and the salvage pathway are able to function from this stage onwards. After treatment with the test compound, the *HPRT*$^-$ mutant cells are selected for using 6-thioguanine (6-TG). *HPRT*$^+$ cells incorporate 6-TG into the DNA and die, and *HPRT* $^-$ cells do not incorporate this toxic analogue into their DNA, and they survive.

Ethyl-methanesulphonate (EMS) and Ethyl-nitrosourea (ENU) can be used as the positive controls in the absence of exogenous metabolic activation (OECD 1997). 3-

Methylcholanthrene, N-nitrosodimethylamine or 7,12-dimethylbenzanthracene can be used as a positive control in the presence of exogenous metabolic activation (OECD 1997).

- To avoid false negative results from the effects of metabolic cooperation, in which wild type HPRT+ cells cross feed killing off TG-resistant mutants, keep cell densities below the threshold at which this occurs. To determine the threshold, plate cells across a series of cell densities keeping 6-TG levels constant. The frequency should not be high enough to yield more than a few colonies. Suspension cells are not prone to this phenomenon and should be feasible at cell densities of 10^5 cells per 96 well plate well.

- For nanotoxicity studies it is advisable to use fresh HAT whenever deciding to stagger plating (used to make large dose-series more manageable), as cells may exhibit delayed or no cell revival or proliferation with free-thaw HAT stocks **(**Doak et al. 2012**)**.

4. METHOD

4.1 Metabolism:

Compounds that require metabolic activation require either an exogenous source such as S9 (treatment time of 3 to 6 hours with the test compound), or a genetically modified cell line such that metabolic activation is endogenous. For example MCL-5 cells are derived from L3 cells, a subpopulation of AHH-1 cells that express a particularly high level of CYP1A1 activity (Crespi et al. 1991; Woodruff et al. 2001). The MCL-5 cell line has also been transfected with two plasmids: one containing two copies of CYP3A4 cDNA and one copy of CYP2E1 cDNA, and a second containing one copy of each CYP1A2, CYP2A6, and

microsomal epoxide hydrolase cDNA (Crespi et al. 1991; Woodruff et al. 2001). Therefore MCL-5 cells stably express all five cDNAs and also have increased levels of CYP1A1 compared to AHH-1 cells, and test compounds that are known to be metabolised by these enzymes can be tested using MCL-5 cells. Genetically modified cell lines that stably express metabolic enzymes can promote more stable and reliable results as there are fewer variables such as pH, osmolality or high levels of cytotoxicity than when adding crude cell extracts of liver, S9.

4.2. *HPRT* Mutant Cleansing

Mammalian gene mutation assays depend upon the ability to quantify mutant cells using selective media. The mutation frequency (MF) at each test concentration is compared to the control MF, and the control is a measure of the spontaneous MF. Therefore, the spontaneous MF should be maintained at a low and stable level within each laboratory. To decrease the spontaneous MF of cultures the number of *HPRT* $^{-}$ mutants are reduced using HAT (Hypoxanthine Aminopterin Thymidine) medium, which inhibits the endogenous *de novo* nucleotide biosynthesis pathway so that the salvage pathway is required for dNTP synthesis for DNA replication. Therefore, cells that are incapable of using the salvage pathway (i.e. *HPRT* $^{-}$ mutants) can no longer divide and undergo cell death.

4.3. Treatment Protocol

Following 'mutant cleansing' the cells are sub-cultured and grown for 24 hours in HT medium. The cells are washed and grown in normal culture medium for 3-4 days to attain sufficient numbers for treatment. This can be a good time to cryogenically freeze down the cells for storage, if for any reason the experiment needs to be carried out at a later date. However, one should be careful that there are no extra days added to the assay as these can cause clonal expansion and the spontaneous MF can be increased. 10ml treatment flasks or 6 well plates are set up and the test chemical is added to the cultures at pre-defined concentrations, which should usually be separated by no more than a factor of between 2 and $\sqrt{10}$, and should also cover a range of high toxicity to little or no toxicity (OECD 1997). There are currently revisions taking place to this guideline, which currently states that "the maximum concentration is based on cytotoxicity then it should result in approximately 10-20% (but not less than 10%) relative survival (relative cloning efficiency) or relative total growth". A typical design of the *HPRT* assay would be testing up to 10mM or 5,000µg/ml (OECD 1997) with a maximum of 1 insoluble concentration, because cells grow

in suspension and precipitate cannot be removed. However, this has recently changed for pharmaceutical testing which follows the guidelines produced by the International Conference on Harmonisation, where the top dose has been "reduced from 10mM to 1mM in order to reduce the number of irrelevant/false-positive *in vitro* findings" (ICH 2011).

Treated cultures are then incubated for 4hours or 24hours depending on the half-life of the compound and whether S9 is added, and each test compound should be prepared and dissolved in the correct solvent using sources of information such as the batch guidelines or the Merck index. A typical design of the *HPRT* assay would be with and without S9 (4 hour treatments) plus a 24 hour treatment without S9.

Each experiment is then carried out in at least duplicate, and each duplicate treatment flask is treated using a stock solution that is prepared each time (i.e. 2 times for a duplicate experiment) from the purchased product, to allow for variation in preparation procedures. *HPRT* assays usually include 4 or more concentrations with duplicate treatments per concentration, or in triplicate if more advanced statistical analysis is required.

Negative controls must be used, and the solvent should not have a significantly different MF than the spontaneous MF. Positive controls must also be used (OECD 1997). Following chemical exposure the cells are centrifuged to remove the test medium, washed and re-suspended in fresh culture medium for mutant expression. For the *HPRT* mutation assay this involves incubation for 13 days. This allows any mutations to become fixed and any existing HPRT proteins/RNA to become degraded. During the phenotypic expression period, the cells are sub-cultured every other day, on days 1, 3, 5, 7, 9 and 11, by centrifugation and re-suspension of the cells in fresh culture medium. Cells can be cryogenically frozen down at this stage which helps when carrying out large dose response studies where large numbers of plates are required per dose. The day of freezing down should be recorded so that the number of expression period days is not altered due to this process. It is not advisable to freeze them down after day 9 as this is close to the time of plating, and we have previously shown this to increase the background mutant frequency. After the phenotypic expression period, the cells are added to 96-well microplates at 40% confluence ($4x10^4$ cells/well for AHH-1 and MCL-5 cells) in culture medium with selection using the toxic analogue 6-thioguanine (6-TG from Sigma UK) at 0.6μg/ml. It is difficult to treat a sufficiently large number of cells (>10^5 cells

per petri dish) to produce statistically powerful assays. Therefore, we make use of a microplate protocol to improve the sensitivity of assay.

Plates are scored for colony formation after 14 days of incubation at 37^0C in humidified incubator with 5% CO_2. The outside wells of the 96-well microplate can dry up due to the long growth period in the incubator, to give false negative colony growth. Special plates can be purchased that have a channel for water which keeps the cells in a humid environment, or

- Scoring colonies of attached cell lines is different from scoring colonies of suspension cell lines, and this should be considered when deciding whether they are defined as viable colonies (e.g. >20 cells diameter) or not (e.g. <20 cells diameter or the cells are dead).

- Adding 6-TG can kill the colonies at a late stage of growth, and therefore you should be careful that you are scoring viable colonies and not dead cells. This can be determined by observing the morphology of the cells. For example dead AHH-1 lymphoblastoid cells are darker and their cell walls are also less circular than viable cells.

other methods can be designed to keep the cells in a humid environment.

For the cloning efficiency (P.E) calculation, 2-200 cells/well can be plated with no selection at each dose.

4.4. Scoring Method

The criteria for colony counting include only scoring colonies of 20+ cells in diameter and ensuring separate colonies are clearly apart, thereby accounting for clonal expansion. This value was defined by us in Swansea and other laboratories may wish to define other scoring criteria. There will also be a large number of dead *HPRT*$^+$ colonies due to 6-TG selection, and these must not be scored as viable colonies.

4.5. Statistical Method

In the presence of metabolic activation and detoxification of a genotoxic compound, the kinetics of DNA-adduct formation is considered first order. A biologically relevant increase in mutation rate which deviates from a linear response may only be observed at dose levels that result in saturation of the DNA repair processes. In order to determine this point of departure (PoD) several statistical tests may be utilised. The Genetic Toxicology Technical Committee (GTTC) quantitative workgroup, formally known as the International Life Science Institute Health and Environmental Sciences Institute (ILSI-HESI) recommend the use of the 1-sided Dunnett's test for data with normal distribution and homogeneous variance, 1-sided Dunnett's T3 for normal distribution and heterogeneous variance and 1-sided Dunn's test for non-parametric data. These tests are available in packages such as SPPS and SAS, but the ILSI-HESI group have recently submitted a package called 'DRSMOOTH' to CRAN, which will allow these tests and more PoD tools to be used on the open-source statistical software R (http://cran.r-project.org/). No observed and lowest observed genotoxic effect levels (NOGEL and LOGEL) can be defined using these approaches for use as PoD metrics for HPRT data with suitable statistical power.

Other PoD methods include bilinear modelling (two lines with different slopes) such as the hockey stick model, which may be used to determine the 'line of best fit' (Lutz and Lutz 2009) and the segmented package available in R (Muggeo 2008; Johnson et al. 2014). A novel package based on mgcv, which provides generalised additive modelling functions based on a penalised regression spline approach with automatic data smoothness function, can also defined as a usable PoD metric called a slope transition dose.

The most well regarded approach for defining a PoD using HPRT data is the benchmark dose approach (Gollapudi et al. 2013; Johnson et al. 2014). Benchmark dose (BMD) analysis can be conducted using PROAST, the dose-response modelling software developed at the National Institute for Public Health and the Environment (RIVM) in the Netherlands (www.proast.nl; version 36.9 (Slob 2002), 2002). BMD analysis can also be conducted using EPA's Benchmark Dose Software (EPA 2013). For continuous datasets such as HPRT mutant frequencies, the $BMDL_{10}$ and the $BMDL1_{SD}$ are recommended (Johnson et al. 2014).

4.6. Clonal Expansion of Mutants and DNA Extraction

Mutant *HPRT*$^-$ colonies are removed from the 96-well microplates using Pasteur pipettes and re-suspended in fresh culture medium. Cultures are maintained and grown to confluence for sufficient numbers of cells for DNA/RNA extraction.

4.7. *HPRT* Mutation Spectrum Analysis

The *HPRT* gene has been characterised largely using the polymerase chain reaction (PCR), with multiplex PCR being used for exon deletion detection i.e. many DNA fragments (i.e. different exons) can be amplified simultaneously, which alongside sequencing can detect smaller mutations (McPherson et al. 1991). The coding region of the human *HPRT* gene is distributed over 39.8kbDNA and contains 9 coding exons. The RNA transcript can also be sequenced using reverse transcriptase PCR, and the resulting mutation spectrum from this technique provides sequence information of mRNA (cDNA) and this is least costly, with regards to both time and money, than multiplex PCR (Thomas et al. 2013).

- Each replicate should have a minimum of 4 x 96-well microplates for 6-TG selection, and 2 x 96-well microplates for cloning efficiency (viability). This can be increased if the experiment is designed to detect smaller changes in MF increase. For example, 100 x 96-well microplates for 6-TG selection and 50 x 96-well microplates for cloning efficiency are required in total for threshold analysis.

- Cloning efficiency is a crucial step in the *HPRT* gene mutation assay. It is important to optimise the number of cells plated for this step (2-200 cells/well) for the particular laboratory and particular cell line.

- For molecular analysis it is imperative to obtain independent mutational events. To ensure this plate replicate cultures at the minimum cell density required for TG-resistance. When harvesting cells harvest a single mutant per replicate.

5. CALCULATIONS

Mutation Frequency and Cloning Efficiency Equations (Furth et al. 1981)

Cloning Efficiency

Cloning Efficiency % (CE) = $-\text{Ln}\ (X_o/N_o) \times 100$

Cell Viability (Relative Cloning Efficiency)

$$\text{Cell Viability \%} = \frac{\text{CE}}{\text{CE of control}} \times 100$$

Mutant Fraction

$$\text{Mutation frequency (MF)} = \frac{-\text{Ln}\ (X_s/N_s)}{-\text{Ln}\ (X_o/N_o)} \times \text{DF}$$

$$\text{Dilution factor (DF)} = \left[\frac{\text{(No. of initial cells per well) Non-selective conditions}}{\text{(No. of initial cells per well) Selective conditions}}\right]$$

X_s= No. of wells without colonies
N_s = Total no. of wells
} Selective conditions

X_o= No. of wells without colonies
N_o = Total no. of wells
} Non-selective conditions

Worked examples:

HPRT$^-$ mutation frequency (MF) calculation for replicate B at 0 µg/ml MMS

$$\text{MF} = \left[\frac{-\text{Ln}\ (1699/1800)}{-\text{Ln}\ (1218/1920)}\right] \times \frac{20}{40000} = 0.0577468 / 0.455115 * 0.0005 = 6.344*10^{-5}$$

If cells have a low cloning efficiency, it can be due to [23]:

1.) A bad batch of 96-well microplates (relatively rare)
2.) A bad batch of horse serum (relatively rare). Make sure you use the same batch of horse serum throughout your experiment.
3.) High pH on the 96-well microplates (common) due to:
    a. Opening the incubator too much during the first 4-5 days.
    b. Low CO_2 setting.

c. Incorrect medium pH (should be 6.8 to 7.0 before adding serum).

4.) Poorly growing cells.

If cells have a high negative control mutant fraction, it can be due to [23]:

1.) An artefact due to low cloning efficiency.

2.) Improper HAT/HT treatment due to:

   a. Thymidine starvation.

   b. Inadequate aminopterin.

3.) Exposure to a mutagen (sunlight).

4.) Inadequate selective agent.

6. ASSAY BENEFITS AND LIMITATIONS

The *HPRT* assay is adapted for use in mammals as well as mammalian cultured cells offering pertinent advantages over microbial tests. These include the genomic organisation being similar to that of humans (and is absent in bacteria) as well as mammalian specific cell metabolism cannot be replicated in bacteria.

As an endogenous gene *HPRT* is transcriptionally active and subject to transcription coupled DNA repair, making it an ideal assay for DNA damage and DNA repair studies alike. Indeed HPRT gene mutation assay detects point mutations, small insertions, frame shifts and small deletions and has been successfully used in mapping mutation hotspots specific to particular genotoxicants. As with any assay there are, however, limitations. *HPRT* is an X-linked gene and thus hemizygous in function. Detection of large deletions, non-disjunction events and chromosomal recombination is very poor due to such events being lethal to the cell and being hemizygous there is no compensation for loss of gene function resulting inevitably in cell death. Negative results in the HPRT assay, for example, were observed when using V79 cells treated with cyclopentenone (Solecki et al. 2013). The authors concluded that large chromosome deletions may have been the predominant form of induced mutations. This hypothesis is supported by investigations showing that oxidative stress is at most weakly mutagenic in terms of point mutations and small deletions, but mutagenic through a mechanism involving large rearrangements (Gille and van Berkel 1994).

Where the intention is to look for non-disjunction and translocation events then use of thymidine kinase (TK) gene as the gene of target would be a suitable alternative and has been reported to have a higher mutation rate relative to HPRT (Johnson 2012).

In animal experiments it is worth remembering that dilution of mutant T-lymphocytes in circulation occurs as peripheral lymphocyte populations are renewed. T-lymphocyte number is also affected by the age of the animal (OECD 2005). As a result, sampling in the spleen (bone marrow may also be used) should be carefully timed to detect the maximum mutant frequency. It is worth noting that published studies typically focus on T-lymphocytes, which prevents identification of mutagenic effects that may arise preferentially in other target tissues.

Different sensitivities of the cell lines used may also play a role in reported discrepancies observed for mutagenicity of several compounds of interest. These variations in cell line sensitivity may be due to the species of origin as well as the relative fidelity of the DNA repair capacity in the cells selected. If cells with reduced repair capacity are utilised for the HPRT assay then false positive data-sets could potentially be the result. Recent examples include nanotoxicity studies whereby conflicting data exists in studies that have utilised the HPRT assay in determining the genotoxic potential of TiO2 and carbon nanotubes (Wang et al. 2007; Huang et al. 2009; Asakura et al. 2010; Doak et al. 2012).

Despite the *HPRT* being ideal in isolating and characterising genotoxic mutants it is not ideal for dose response analysis given the number of replicates and thus number of cells and doses required. Latest advances have seen the introduction of HPRT activity assays that may be used as an alternative. Such assays which include PRECICE® HPRT Assay Kit from Novocib that allow the investigator to monitoring of HPRT activity in a convenient 96-well plate format using spectrophotometric analysis, after treatment with the compound of interest. These authors would like to see flow cytometric approaches for novel *in vitro* mutation tests coming forward, and they hold out much promise for things such as the *in vitro* PigA assay where 10,000 cells per dose can be scored in a matter of minutes from a single tissue culture flask, compared to hours of scoring in ~100 x 96-well plates.

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