Protocol for *HPRT* mutagenesis analyses

Contributed by Ray Monnat, April 2000; updated July 2007, April 2009.

Introduction: The following is a series of notes to help you establish and use the HPRT mutagenesis system to generate quantitative and molecular data on mutagenesis in human cells. Many of the purely technical aspects of using this system have been lost to the sands of time, so the most practically useful advice is summarized here. You are also referred below to several references for additional practical background on using this system to generate mutation data in your cell types of choice.

Getting started

1. Determine the kill curve for TG in the cell line of interest

Plate 10,000 cells/60 mm dish or plate well and grow in the presence of increasing concentrations of 6-thioguanine for 7 - 10 days. The usual concentration that will kill everything except preexisting TG-resistant mutants is 1 - 10 μg/ml (6 - 60 μM) in both attached and suspension cells.

notes:

- TG stocks should be stored at -20/-80ºC and diluted into media just before use. Storing stocks, especially diluted stocks or '+TG' media for long periods of time before use is not recommended.
- The stability of 6-TG is easy to confirm by spectrophotometry: see the protocol for Thioguanine Medium Preparation for specifics and a comparison trace from Fenwick's chapter reference. The stability of 6-TG in different culture media hasn't been established but is unlikely to be a problem if the media is changed every 5-7 days;

2. Determine whether high endogenous TG-resistance is a problem in the cell line(s)

Plate 100,000-200,000 cells in a 100 mm dish in the presence of at least 30 μM 6-TG and continue the culture for at least 14 days with refeeding. The frequency shouldn't be high enough to yield more than a few colonies.

notes:

- A potential confounder and source of false negative results is 'metabolic cooperation', in which wildtype (HPRT+) cells cross-feed and kill off pre-existing TG-resistant mutants. This can be avoided by keeping cell densities below the threshold at which this starts to occur. Suspension cells don't seem prone to this phenonemon, and thus can be cultured at surprisingly high densities (e.g., 10^5 cells per 96 well plate well) without suppression of TG-resistant mutant recovery.

- The maximum allowable cell density for selection can be determined in a mixing experiment in which the recovery of a known number of pre-existing TG-resistant mutants is measured under conditions of increasing cell density. Less than ~200,000 cells/100 mm dish is a safe limit for most human fibroblast-like cells.
- If background TG-resistant frequency is high (say $1/10^{3-4}$ or even higher) the first thing to do is check for *Mycoplasma* infection of the culture by DAPI staining and fluorescence microscopy. If the culture is *Mycoplasma*-negative, it is possible to clean existing TG-resistant mutants out of the system with HAT counter-selection medium and then measure new TG-resistant mutants as they emerge. However, be forewarned that HAT counterselection has its own problems and in many cases cells must be weaned off of HAT selection by growth in HT medium to avoid high levels of cytotoxicity due to a lag in recovery from aminopterin block. A recipe for HAT medium prep is attached that's been experimentally validated.
- A second problem with HAT selection is that you may have to deal with expression or phenotypic lag problems before you can get a reliable estimate of the effect of genotype or treatment on TG-resistant mutant generation. See the standard system references listed below for additional discussion and advice on this.
- Phenotypic lag is also a problem in induced mutagenesis experiments, where it may take several cell doublings for residual HPRT activity to decay to the point where TG killing becomes efficient and you can begin to recover newly induced HPRT mutant cells. If you need accurate numbers, you may wish to do a time course that determines and plots the increase in fraction of TG-resistant mutants as a function of time or cell doublings after treatment.

3. Do the experiment

Plate comparable numbers of cells or cells with and without treatment at densities that allow for the determination of colony-forming efficiency and to recover TG-resistant mutants. If you're generating a dose-response curve you should plate duplicates at a minimum of three concentrations. If you want a frequency in the absence of treatment do triplicate plates for each cell line.

notes:

- **Reread the advice and caution above regarding metabolic cooperation**: Make sure you're in an efficient range of TG and cell density to allow the optimal recovery of TG-resistant mutants. If you're not sure, do the mixing experiment!
- **If the aim is numbers**: You need appreciable numbers (>10 TG-resistant colonies/ experimental arm) to get a reliable estimate of *mutant frequency* and how it might be changing as a function of genotype or treatment. Since the background frequencies of TG-resistant mutants can be low ($\sim 10^{-6}$) this can entail plating a large number of plates and then pooling numbers from several plates. My suggestion is do a pilot first to determine where to start, then correct or add depending on whether the frequency is high or low and whether you have to

factor in cell killing in response to a treatment. If you know the number of cells you start and end with, the CFE and the number of mutants you recover in several replica cultures you can also calculate a *forward mutation rate* to HPRT-minus.

- **If the aim is molecular analysis**: You want indisputably *independent* mutational events. The best way to get these is to grow multiple small replicate cultures to the point at which you should be getting TG-resistant mutants, and then plate each replicate under selection conditions. Harvest a *single* mutant per replicate for molecular analyses.
- **Use constant selection in both types of protocol**: Keep constant TG selection on during the isolation and expansion of putative mutant colonies. During early experiments we observed a serious confounder effect in which wildtype cells could survive but not grow in the presence of TG and then could out-compete TG-resistant mutants if the expansion of putative TG-resistant clones was done in the absence of TG. It is not clear how widespread this phenomenon is.

Standard HPRT system references:

note: The following are a series of hard to find but useful, technically oriented *HPRT* system references.

1. Jacobs, L. & DeMars, R. Chemical mutagenesis with diploid human fibroblasts, in *Handbook of Mutagenicity Test Procedures* 2nd Ed. (eds Kilbey, B.J., Legator, M., Nichols, W. & Ramel, C.) Elsevier Science Publishers, Amsterdam, pp.321-356 (1984).

2. Fenwick, R. G. The HGPRT system, in *Molecular Cell Genetics* 1st Ed. (ed Gottesman, M.) Wiley, New York, pp.333-373 (1985).

3. Thilly, W. G., DeLuca, J. G., Furth, E. E., Hoppe IV, H., Kaden, D. A., *et al.* Gene-locus mutation assays in diploid human lymphoblast lines, in *Chemical Mutagens* 6 (eds de Serres, F.J. & Hollaender, A.) Plenum Press, New York, pp.331-364 (1980).

Additional HPRT system references:

1. There is a chapter on HPRT deficiency and Lesch-Nyhan syndrome in successive editions of *Stanbury's The Molecular and Metabolic Basis of Inherited Disease.* This chapter is a good place to start for background on clinical, molecular and historical background on HPRT deficiency and related purine metabolic defects.

2. Szybalski, W. (1992) Use of the HPRT gene and the HAT selection technique in DNA-mediated transformation of mammalian cells: first steps towards developing hybridoma techniques and gene therapy. *BioEssays* 14:495-500. (early history of the *HPRT* system)

Thioguanine Medium Preparation

6-Thioguanine (TG) and other purine analogues (e.g., 8-azaguanine) select for HPRT-minus cells. Only TG should be used for standard forward *HPRT* mutation selections. Buy a large lot and prepare standardized stocks to freeze. Thaw and use aliquots as needed. Check the initial large lot by spectrophotometry and by generating a killing curve for any cell line of interest to determine the TG concentration needed to perform clean biological assays.

1. Prepare TG stock (5-10 mg/ml, 30-60 mM) by dissolving 6-TG powder (Sigma) in smallest possible amount of fresh 0.1 N NaOH, then dilute up to volume with sterile water.
2. Filter sterilize through a 0.22 µm filter and store aliquots at -20°C.
3. Thaw working stock and use immediately or within a week of thawing. TG deteriorates rapidly when stored at 4°C.
4. The absorption spectrum of TG over 220-340 nm can be used to gauge the quality of TG stock solutions. Dilute stocks 10- to 100-fold with water, blank the spectrophotometer with water, and measure the spectrum. A good stock has a 320:260 ratio of greater than 2.5 (see Fenwick Figure 13.1 below). The extinction coefficient could also be used to determine the TG concentration, but a biological check of activity is the most important measure of activity or effective concentration.

8-azaguanine **6-thioguanine**

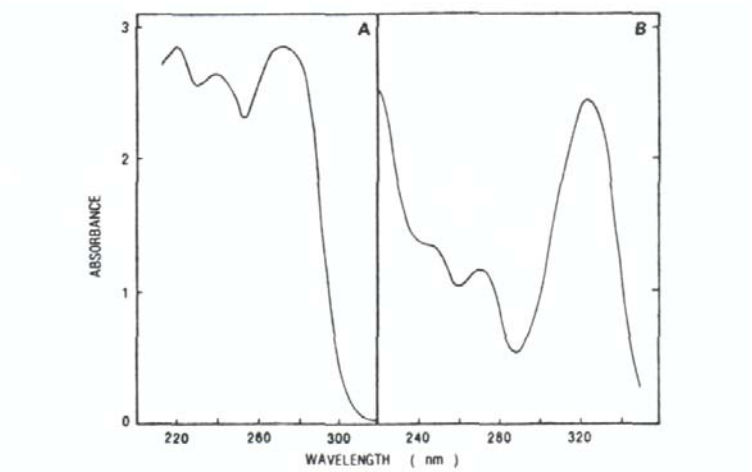

Figure 13.1. Absorption spectra of selective agents. Stock solutions of 140 µM 8-azaguanine (A) and 27 µM 6-thioguanine (B) prepared in 0.01 *N* KOH were diluted 10-fold with water prior to measurement of their absorption spectra.

"HAT" Medium Preparation

"HAT" medium selects for HPRT(+) cells. The components are **h**ypoxanthine, **a**minopterin, and **t**hymidine, hence the acronym "HAT". Updated from Monnat/Leone, May 1985.

1X "HAT" is:

100µM	hypoxanthine	(H)
1µM	aminopterin	(A)
20µM	thymidine	(T)

The easiest way to use HAT medium is with commercially available 100X HAT (from Sigma or GIBCO) although the concentrations of the components can vary. If a large amount of 100X HAT is needed, the following 100X stocks of hypoxanthine plus thymidine and of aminopterin can be prepared.

100X Hypoxanthine-Thymidine (HT) stock (100ml):

	(FW)	(M)	(volume)	=	wt added/100ml
Hypoxanthine:	(136.1)	(0.01)	(0.1)	=	136 mg/100ml
Thymidine:	(242.2)	(0.002)	(0.1)	=	48.4 mg/100ml

Dissolve hypoxanthine by stirring in 98ml deionized water at 45°C for approx 1 hr. Cool and add thymidine, stirring to dissolve. Adjust volume to 100ml and filter sterilize. Store as 1 ml aliquots at -80°C.

100X Aminopterin (A) stock (100ml):

	(FW)	(M)	(volume)	=	wt added/100ml
Aminopterin:	(440.4)	(0.0001)	(0.1)	=	4.4 mg/100ml

Dissolve aminopterin in a few ml of sterile 0.1N NaOH, then dilute up to 98 ml with deionized water. Adjust pH to 7.0 with HCl and adjust final volume to 100 ml with deionized water. Filter sterilize and store in 1 ml aliquots at -80°C. Protect from light.

"HAT" medium compounding:

Add 1 volume each of 100X HT and 100X A stocks to 100 volumes of the medium of your choice. Label medium as "+HAT".