The *gpt* Assay of *gpt* delta Transgenic Mouse

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The *gpt* gene of *E. coli* encodes guanine phosphoribosyltransferase involved in purine salvage pathway of *E. coli*. This enzyme catalyzes phosphoribosylation of guanine, which is the obligatory step for the incorporation of guanine to DNA. However, this enzyme also phosphorybosylates 6-thioguanie (6-TG), which is toxic to cells when it is incorporated into DNA. Thus, *E. coli* cells having wild-type *gpt* gene cannot survive on plates containing 6-TG because of the toxicity. Only *gpt* mutants can form colonies on plates containing 6-TG. It is a practicable method to positively select *gpt* mutants of *E. coli.* In addition, the coding region of the *gpt* gene is 456 bp, which is convenient for the identification of mutation by DNA sequencing.

The transgenic mice *gpt* delta carry λ phage EG10 as a transgene. The phage DNA carries a linearized plasmid, containing *E. coli gpt,* chloramphenicol acetyltransferase (CAT) and the replication origin of plasmid pBR322, which is flanked by two-direct repeat sequences of *loxP*. λEG10 is rescued from genomic DNA of mice as phage particles by *in vitro* packaging reactions. When the rescued phages are infected to *E. coli* strain YG6020 expressing Cre recombinase, the plasmid region is efficiently excised from the phage DNA, circularized and propagated as multi-copy-number plasmids carrying *E. coli gpt* and CAT. *E. coli* cells harboring the plasmids carrying mutant *gpt* gene and CAT can be positively selected as bacterial colonies arising on plates containing 6-TG and chloramphenicol (Cm). The number of rescued phages can be determined by plating the cells on plates containing Cm alone. The mutant frequency of *gpt* is calculated by dividing the number of colonies arising on plates containing 6-TG and Cm with that of colonies arising on plates containing Cm alone.

The following is the protocols for the *gpt* assay of *gpt* delta transgenic mouse. The protocol for *in vitro* λ phage packaging reactions is not provided because it is mostly the same as that used for other transgenic mouse mutagenicity tests using λ phage shuttle vectors.

The *gpt* Assay of *gpt* delta Transgenic Mouse ------------------(cover page) p1

1. Detection of mutants and calculation of mutant frequency --------- p2
2. Confirmation of Mutant Phenotypes and Storage of Mutant Cells ------ p4
3. Preparation of reagents and medium --------------------------- p5
4. Other reagents and materials ----------------------------------- p7

1. Detection of mutants and calculation of mutant frequency

To calculate the mutant frequency of *gpt*, conduct Titer (survival) assay and 6-TG (mutant) selection.

<Preparation of Bacteria>

1. Make an overnight culture of the master stock of *E. coli* YG6020 with LB+maltose broth containing 25 µg/ml Km.

2. Subculture the overnight culture by diluting 1:40 in LB+maltose broth containing 25 µg/ml Km. Incubate in 37°C water bath with vigorous agitation until OD600 reaches 1.0 (for about 3 hours).
   <note> About 5 ml of YG6020 culture is used for the assay per one packaged sample.

3. When the culture reaches OD600=1.0, recover the cells by centrifugation at 4000g for 10 minutes at 4°C.

4. Resuspend the pellets with 1/2 vol. of LB+10 mM $MgSO_4$ broth (OD600=2, theoretically). Store the cell suspension at 4°C until use.

<Titration (Survival)>

1. After in vitro packaging reactions, each packaged phage sample is adjusted to the final volume of 300 µl with SM buffer. Mix 5 µl of the packaged phage suspension with 495 µl of LB+10 mM $MgSO_4$ broth to make a 100-fold diluted suspension.

2. Add 10 µl of the diluted suspension to 200 µl of the 2-fold concentrated YG6020 culture in two sterile small glass tubes (see more detail in "Other reagents and Materials") and mix gently.
   <note> To get a better estimate for the survival, the experiments should run double, *i.e.*, two tubes should be used for one estimation.
   <note> YG6020 culture should be mixed well just before use.

3. Incubate at 37ºC for 20 min without shaking for adsorption of phages to *E. coli* cells.

4. Incubate at 37ºC for 30 min with shaking for conversion of phage DNAs to plasmids.
   <note> We shake with speed of 300 r.p.m.

5. Add 2.5 ml of soft agar, mix by vortex and pour on M9+Cm agar plates.
   <note> We use 9 cm diameter plastic dishes.
   <note> Freshly prepared plates should be used.

6. Incubate at 37°C for 3 days.

7. Count the number of Cm^r colonies.

[Titer = Number of Cm^r colonies (average of 2 plates) x Dilution Factor]
Dilution Factor = 3000*
* Because each titer plate contains 0.1 µl of the original packaged sample (300 µl).

<6TG selection (Mutant)>

1. After *in vitro* packaging reactions, each packaged phage sample is adjusted to the final volume of 300 µl with SM buffer as described in the section of "Titer". Add 60 µl of the packaged suspension to 200 µl of the 2-fold concentrated YG6020 culture in 5 sterile glass tubes and mix gently.
   <note> Five sterile glass tubes are used to test one sample (300 µl = 60 µl x 5 plates).
   <note> YG6020 culture should be mixed well just before use.

2. Incubate at 37ºC for 20 min without shaking for adsorption of phages to *E. coli* cells.

3. Incubate at 37ºC for 30 min with shaking to convert phage DNAs to plasmids.
   <note> We shake the tubes with shaking speed of 300 r.p.m..

4. Add 2.5 ml of 6TG soft agar, mix by vortex and pour on M9+Cm+6TG agar plates.
   <note> We use 9 cm diameter plastic plates (5 plates/sample).
   <note> Freshly prepared agar plates should be used.

4. Incubate at 37°C for 72 hours* and count the number of colonies.
   <note> Total number of colonies per 5 plates are calculated as a number of $6TG^r$ mutants per one packaging (300 µl).
   <note> Very small colonies appeared on the plates after incubation for 4 days but not 3 days should not be counted.

*Care must be taken to correctly count the *gpt* mutant colonies on 6TG agar plates. The *gpt* mutant colonies are often small and are not easily distinguished from small needle-shape crystals (maybe 6TG) which appear on the 6TG plates after incubation for 3 days. Hence we usually mark the possible mutant colonies with pen at 72 hrs incubation and confirm that they are colonies but not crystals in the following day. At day 4, very small colonies start to appear on 6TG plates. Most of these slowly-appearing colonies are not real *gpt* mutants when they are examined in the isolation and confirmation test as described in the next section.

[Number of $6TG^r$ mutants = total number of $6TG^r$ colonies on 5 selection plates]

2. Confirmation of Mutant Phenotypes and Storage of Mutant Cells

<Confirmation of mutant phenotypes>

The mutant colonies on the 6TG plates are picked up by sterile toothpicks. After the tip of toothpick is rinsed well with 50 µl of 1/15M Na-K buffer, suspended cells are streaked on both a titer plate containing 25 µg/ml of Cm and a selection plate containing 25 µg/ml of Cm and 25 µg/ml of 6TG. Plates are incubated for 3 days at 37°C to confirm the 6TG-resistant phenotype.
<note> The fresh plates used for the 6TG selection can be stored at 4C for the confirmation test.
<note> Streak on the M9+Cm+6TG plate at first, then on M9+Cm plate.

<Calculation of mutants frequency>

The mutant frequency is calculated by dividing the total number of $6TG^r$ and Cm^r colonies by the total number of Cm^r colonies rescued.

[Mutant Frequency] = [Number of $6TG^r$ mutants] / [Titer]

Number of $6TG^r$ mutants: Number of $6TG^r$ and Cm^r colonies on the 5 M9+Cm+6TG plates (after confirmed)
Titer: Number of colonies per plate on the M9+Cm plate x Dilution Factor
Dilution Factor = 3000

<Storage of mutant cells>

The 6TG-resistant colonies are inoculated into 2 ml of LB medium containing 25 µg/ml of Cm and cultured overnight at 37°C. Cells are collected by centrifugation and supernatant is discarded. Obtained bacterial pellet is stored at -80°C for the sequencing of *gpt* gene.

3. Preparation of Reagents and Media

Stock solution (reagents, media)

Reagent	Storage
LB broth 20% (w/v) Maltose 10 x M9 salt 50% (w/v) Glycerol 1M $MgSO_4$ 1M $CaCl_2$ 1% (w/v) Thiamine 10 mg/ml Amino acids (Pro, Leu, Ile)	store each reagent at room temperature after sterilize by autoclave or filtration.
25 mg/ml Kanamycin	store at -20 °C.
25 mg/ml Chloramphenicol	EtOH solution. store at -20°C.
25 mg/ml 6-Thioguanine	DMSO solution. It should be freshly prepared.

6-thioguanine : TCI (Tokyo Kasei), 1g, T0212

Plates

[BOTTOM] (1.5% agar*) 30 ml/plate	[TOP] (0.6% agar*) 2.5 ml/plate
M9+Cm plate	soft agar
M9+Cm+6TG plate	6TG soft agar

*BACTO AGAR : Difco, 1lb, 0140-01

Preparation of Plates

1. M9+Cm +/- 6TG plate (Cm 25μg/ml, 6TG 25 μg/ml)

Agar	15 g
H_2O	900 ml

Autoclave with stirrer bar.
Allow the solution to cool to 60C, and add supplements.

10 x M9 salt *	100 ml
50% (w/v) Glycerol	20 ml
1M $MgSO_4$	2 ml
1M $CaCl_2$	100 μl
1% (w/v) Thiamine	500 μl
Amino acids	4 ml
(Amino acids : 10 mg/ml Pro, Leu, Ile for YG6020)	
25 mg/ml Chloramphenicol (in EtOH)	1 ml
25 mg/ml 6-Thioguanine (in DMSO)	1 ml

Pour 30 ml/plate (9 cm diameter).

* 10 x M9 salt

Per liter

$Na_2HPO_4 \cdot 7H_2O$	128 g
KH_2PO_4	30 g
NaCl	5 g
NH_4Cl	10 g
H_2O	

Autoclave.

2. Soft agar (0.6% (w/v) NaCl, 0.6% (w/v) agar)

Agar	0.6 g
NaCl	0.6 g
H_2O	100 ml

Autoclave.

<note> Autoclave for sterilization (120ºC, 20 min), and keep it solid at room temperature. Just before using, melt again by short-time autoclaving (120ºC, 5min) and shake well. Keep it in water bath at 50ºC before and during use.

3. 6TG soft agar (25 μg/ml 6TG, 0.6% (w/v)NaCl, 0.6% (w/v) agar)

Add 0.1% vol. of 6TG solution (25 mg/ml in DMSO) to soft agar in 50ºC water before use, and mix well.

4. Other Reagents and Materials

1. LB+maltose broth

    Add 1/100 vol. of 20%(w/v) maltose solution to LB broth (final conc. 0.2%) after sterilization.

2. LB+10 mM $MgSO_4$ broth

    Add 1/100 vol. of 1M $MgSO_4$ solution to LB broth (final conc. 10mM) after sterilization.

3. 1/15 M Na-K buffer (pH 7.4)

    Per liter

H_2O	
Na_2HPO_4	7.57 g
KH_2PO_4	1.82 g

    Autoclave.

4. Test tubes (sterilized)

    small tubes (12.3 x 75 mm)

5. Sterile plate (9 cm diameter)

    EIKEN KIZAI,m CO., LTD., code. No. AK2000

6. Incubator (set at 37ºC)

7. Shaker for small tubes (set at 37ºC)

    Environmental Incubator Shaker
    New Brunswick Scientific Co., Ltd.
    Edison N.J., USA

8. Spectrophotometer

9. Centrifuge

10. Freezer (-80ºC)