Evaluation of an Hprt-Luciferase Reporter Gene on a Mammalian Artificial Chromosome in Response to Cytotoxicity

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ABSTRACT

Background Hypoxanthine guanine phosphoribosyltransferase (Hprt) is known as a house-keeping gene, and has been used as an internal control for real-time quantitative RT-PCR and various other methods of gene expression analysis. To evaluate the Hprt mRNA levels as a reference standard, we engineered a luciferase reporter driven by a long Hprt promoter and measured its response to cytotoxicity.

Methods We constructed a reporter vector that harbored a phiC31 integrase recognition site and a mouse Hprt promoter fused with green-emitting luciferase (SLG) coding sequence. The Hprt-SLG vector was loaded onto a mouse artificial chromosome containing a multi-integrase platform using phiC31 integrase in mouse A9 cells. We established three independent clones.

Results The established cell lines had similar levels of expression of the Hprt-SLG reporter gene. Hprt-SLG activity increased proportionately under growth conditions and decreased under cytotoxic conditions after blasticidin or cisplatin administration. Similar increases and decreases in the SLG luminescent were observed under growth and cytotoxic conditions, respectively, to those in the fluorescent obtained using the commercially available reagent, alamarBlue.

Conclusion By employing a reliable and stable expression system in a mammalian artificial chromosome, the activity of an Hprt-SLG reporter can reflect cell numbers under cell growth condition and cell viability in the evaluation of cytotoxic conditions.

Key words gene reporter; hypoxanthine phosphoribosyltransferase; luciferase; mouse artificial chromosome; reference standards

House-keeping genes have been routinely used as internal controls for normalization in gene expression analysis.1–3 Hypoxanthine guanine phosphoribosyltransferase (Hprt), a nucleotide metabolizing enzyme, is such a house-keeping gene, and has been utilized in many studies of gene expression as a reference standard. As we compared the gene expression inductions by the compounds (mostly drugs) of internal control genes, using the Open TG-GATEs (Toxicogenomics Project-Genomics Assisted Toxicity Evaluation System), HPRT-gene presented the least variation.4 Although it has been widely used as a standard in real-time quantitative reverse transcription PCR (RT-qPCR), the Hprt promoter/enhancer has not been extensively analysed.5, 6

Luciferase assay systems enable the real-time monitoring of gene expression in living cells. We used the green-emitting luciferase (SLG) from Rhagophthalmus ohbai as a reporter gene in this study.7 Reliable expression systems are needed for the evaluation of in vitro gene analysis, but transgene expression in various cell lines established by random genomic integration using conventional methods can be unstable or non-uniform due to gene silencing. Mammalian artificial chromosome technology has been developed to overcome this problem. It has been demonstrated that human artificial chromosomes (HACs) and mouse artificial chromosomes (MACs) are independently retained in host cells and provide stable expression of transgenes.8, 9 In addition to these characteristics, features that allow cell-to-cell transfer of HACs and MACs by microcell-mediated chromosome transfer (MMCT) have shown potential for...
gene therapy application as well as for gene analyses in various situations. A mouse A9 cell line, derivative of mouse fibroblast L cells used for toxicity testing, was useful for a donor cell of MMCT.14

The construction of larger promoter/reporter vectors requires the handling of large DNA regions, which is difficult with common cloning approaches. The bacterial artificial chromosome (BAC) recombineering method is a powerful tool for the manipulation of long DNA fragments.15, 16 Because recombination takes place in bacteria by employing intrinsic bacterial/phage machinery, large vector construction can be achieved without complicated cloning steps. It has been reported that a mouse CD40L gene vector constructed using BAC recombination showed functional expression from a HAC.17

Phage integrases, enzymes that integrate DNA into a bacterial host genome, have been reported to work in mammalian cells.18, 19 PhiC31, R4, TP901l and Bxb1 integrases mediate efficient site-specific recombination in mammalian cells, and transgenesis in mice was also reported by pronuclear injection of phiC31 integrase.20–24 The multiple integrases (MI) system on an artificial chromosome (MI-HAC/MI-MAC), an application of mammalian artificial chromosome technology, was developed for loading gene(s) onto HACs and MACs. The MI platform has five gene loading sites for distinct recombinase/phage integrases.25 By using this recombinase-mediated MI system, targeted recombinant cells can be obtained at high efficiency and these recombinants retain stable transgene expression compared with the random integration method.25 Recently, it has been shown that transchromosomic mice were generated in fewer steps by direct use of mouse embryonic stem cells harboring MI-MAC.26

Here, we constructed a long Hprt-promoter/luciferase reporter vector using a BAC recombineering method and loaded it onto the MI-MAC system. We confirmed luciferase activity that was proportionate with cell numbers in established Hprt-luciferase cell lines.

**EXPERIMENTAL PROCEDURES**

**Vectors**

The inspB4ins2 vector is described elsewhere.26 The vector has two insulator cassettes consisting of repetitive 5'-DNaseI hypersensitive site 4 (HS4) elements from chicken beta-globin to prevent promoter interference from neighboring regions. The PPAC ori km vector was modified from the pPAC4 vector (Children's Hospital Oakland Research Institute, Oakland, CA). The BstEII/AciI region was replaced by a linker sequence containing a multiple cloning site (McaTI, FseI, Pmel and AvrII). pCAG-phiC31 expresses a bacterial phiC31 integrase optimized for mammalian codon usage and is driven by the CAG promoter.

**Hprt promoter cloning by BAC recombineering**

A detailed flow diagram for the construction of phiC31neoHprt-SLG is described in Fig. 1 and Table 1. Briefly, the locus-specific homology arm (white boxes in Fig. 2A) of the Hprt promoter region was synthesized for the retrieving 20 kb BAC fragment. The arm was ligated into the inspB4ins3 vector, and then the coding sequences of the *Rhagophthalmus ohbai* luciferase gene, SLG (pSLG-test vector, Toyobo, Osaka, Japan) was inserted. Additionally, a phiC31neo module (the phiC31 integrase attB site and a neomycin resistance gene cassette) was also ligated into the inspB4ins3 vector. The vector was digested by AciI and AvrII and then ligated into PPAC ori km. The Hprt gene promoter region was retrieved by gap-repair from a BAC clone (B6Ng01-126E09; Riken, Tokyo, Japan) into the MI artificial chromosome (PAC) vector using *E. coli* strain DY380.13 Clones were selected at 32 °C on LB agar containing kanamycin. To check whether the clones were precisely retrieved by gap-repair of the promoter arms, the clones were amplified by PCR, and the vector was confirmed by restriction enzyme digestion.

**Cell culture and compounds**

Mouse A9 (MI-MAC) cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 10% FBS at 37 °C.26 Blasticidin (InvivoGen, San Diego, CA) and cisplatin (Wako, Osaka, Japan) were diluted with culture medium at the time of use.

**Establishment of Hprt-SLG cells**

Principles for recombinase-mediated integration using the MI-MAC system are described elsewhere.25, 26 The phiC31neoHprt-SLG PAC vector was purified using a Large Construction Kit (Qiagen, Hilden, Germany). A9 MI-MAC cells were co-transfected with phiC31neoHprt-SLG and pCAG-phiC31 using Lipofectamine 2000 (Invitrogen) for 6 h (Fig. 2B). Twenty-four hours after transfection, cells were expanded for 24 h and then selected with 600 μg/mL G418 (Invitrogen). Surviving colonies were picked and recombination checked by genomic PCR analyses.

**PCR analyses**

Amplified regions and primer sequences for genomic PCR are described in Fig. 2C and Table 2, respectively. All PCR reactions were performed with KOD FX neo polymerase (Toyobo) under the following conditions.
RepeatMasker to avoid repetitive elements, which
locations of the Hprt retrieving arms were determined
operations for each step are described in Table 1. The
and phiC31neo module (Fig. 1, steps 1 to 5). Detailed
 Avec inspB4ins2: insulator sequence (HS4), retrieving arm, SLG luciferase
ponents were sequentially ligated into inspB4ins2: insu-
ter sequence (HS4), retrieving arm, SLG luciferase
To develop a retrieving vector, the following compo-
Construction of the Hprt-SLG reporter vector
RESULTS

Fluorescence in situ hybridization (FISH) mapping
FISH analysis was performed using a standard proto-
col. Metaphase nuclei from established cell lines (A9
Hprt-SLG cells) were spread on slides. Biotin-labeled
Hprt-SLG vector and digoxigenin-labeled mouse minor
satellite DNA were prepared as hybridization probes.
To suppress background signals, a fifty-fold amount of
non-labeled mouse Cot-1 DNA was added during hy-
bridization. Chromosomal DNA was counter-stained
with DAPI-Fluoromount-G (Southern Biotechnology
Associates, Birmingham, AL). Fluorescence images
were captured by Metafer, and analyzed with ISIS (Carl
Zeiss, Oberkochen, Germany).
Luciferase and cell viability assays
A9 Hprt-SLG cells were seeded at $5 \times 10^4$ cells per well
in a 96-well micro-clear bottom black plate (Greiner,
Kremsmünster, Austria) 24 h prior to compound addi-
tion. After culture for 72 h with blasticidin (0–10 μg/mL)
or cisplatin (0–20 μM), cells were washed twice with
PBS and then subjected to the following analyses. Lucif-
erase activity was measured with a Phelios luminometer
(Atto, Tokyo, Japan) using Tripluc assay reagent (Toyobo).
A cell viability assay was performed using alamarBlue
(ABDSerotec, Oxford, UK) according to the manufac-
turer’s instructions and an Infinite F500 fluorescent plate
reader (Tecan, Männedorf, Switzerland). Three independ-
ent wells were used to determine the Luciferase activi-
ty.

**RESULTS**

**Construction of the Hprt-SLG reporter vector**

To develop a retrieving vector, the following compo-
ents were sequentially ligated into inspB4ins2: insu-
lator sequence (HS4), retrieving arm, SLG luciferase
and phiC31neo module (Fig. 1, steps 1 to 5). Detailed
operations for each step are described in Table 1. The
locations of the Hprt retrieving arms were determined
by RepeatMasker to avoid repetitive elements, which
are known to be deleterious for subsequent BAC re-
trieving (Fig. 2A white boxes). The module contain-
ing all components was transferred to a PAC vector
backbone (Fig. 1, steps 6 to 8), and the resulting PAC
vector was used as a retrieving vector. We successfully
retrieved 20 kb of Hprt promoter from the BAC clone
to give phiC31neoHprt-SLG (Fig. 1 step 9). The large 20-
kb promoter/enhancer region of Hprt was successfully
recloned into an expression vector for Hprt gene expres-
sion using BAC recombineering method.

**Vector transfection into A9 MI-MAC cells**

Figure 2A and B represent a genomic map of the mouse
Hprt gene and schematic map of the MI-MAC and
phiC31neoHprt-SLG vector array, respectively. The
retrieved phiC31neoHprt-SLG vector was co-trans-
fected into A9 MI-MAC cells with a phiC31 integrase
expression vector (Fig. 2B), and 32 candidate colonies
were isolated. We performed sequential PCR analyses
of these clones to clarify the relationship between total
isolated colony numbers and accurate integration in
each step. Figure 2C shows a post-integration map at
the phiC31 site on the MI-MAC and the regions ampli-
fied by PCR using the primer sets in Table 2. All PCR
analysis results are summarized in Table 3. First, 26 of
32 clones were 5'-junction-PCR-positive (#2–11, #13–15,
#17, #19–23 and #25–31). Among 16 of these clones (#2,
5, 6, 9, 10, 13, 15, 17, 19, 21, 22, 26–28, 30 and #31) 10
were positive for the 3'-junction PCR (#2, 10, 13, 15, 17,
21, 22, 26, 27 and #30). Subsequently, only four clones
(#2, 17, 21 and #30) were positive for the 3'-6 kb PCR
that included the SLG reporter element, and then #21
cloned was excluded by failure to amplify the 3'-15 kb
fragment (LP-15 in Table 3 and Fig. 2C). We, therefore,
tained three cell lines (#2, #17 and #30; Hprt-SLG
cells) that integrated the entire vector region from the
16 clones examined. These clones produced almost the
same levels of luciferase activity (Fig. 3A), and FISH
analysis showed Hprt-SLG signals on the MAC (Fig.
3B). Thus, we presumed that these lines had uniform re-
porter gene expression from the MI-MAC.

**Evaluation of Hprt-SLG luminescence compared
with cell numbers**

To verify luminescence linearity of Hprt-SLG cells,
we seeded clone #17 at various densities from 1,000 to
40,000 cells in a 96-well black plate and performed a
luciferase assay after 2 h. Luciferase activity showed lin-
er luminescence profile was mostly consistent with cell vi-
ability measured with an alamarBlue cell viability assay
(Fig. 4A upper panel, blue line). Similar to the MTT as-

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say, the Alamar Blue reagent detects reduced substrates in response to metabolism in living cells. 27 We also seeded 10,000 cells, and measured luciferase activity every 24 h up to 72 h. The correlation of luminescence and living cells showed an almost linear ratio through the incubation period (Fig. 4A lower panel). According to these results, we considered that luminescence intensity of Hprt-SLG reflected living cell numbers under conditions of growth. To assess the reporter response under toxic conditions, we administrated cytotoxic compounds to clone #17. Treatment with blasticidin (Fig. 4B upper panel) or cisplatin (Fig. 4B lower panel) for 72 h decreased SLG activity in a dose-dependent manner. Next, we estimated cell viability using the alamarBlue cell viability assay. We found a positive correlation between SLG activity and cell viability using both blasticidin and cisplatin. We also obtained similar results using clones #2 and #30 (data not shown).

**Fig. 1.** Construction of phiC31neoHprt-SLG.
Flow chart of PAC vector construction for BAC retrieving. Arrows indicate the sequential steps via the operations described in Table 1. The retrieving vector is composed of an Hprt homologous arm-SLG sequence flanked by HS4 insulators and a phiC31neo cassette. BAC, bacterial artificial chromosome; Hprt, hypoxanthine guanine phosphoribosyltransferase; HS4, DNase I hypersensitive site 4; PAC, PI artificial chromosome; SLG, green-emitting luciferase.
Table 1. Operations for constructing the phiC31neoHprt-SLG PAC vector

| Steps in Fig. 1 | Operation | Fragment/digestion (origin) | Insert site |
|-----------------|-----------|----------------------------|-------------|
| 1 to 2          | Fill-in/self ligation | BamHI |                         |
| 2 to 3          | Add HS4 fragment | NheI-AvrII (insP4ins2) | AvrII |
| 3 to 4          | Add arm fragment | BglII-BamHI (synthetic gene arm) | BamHI |
| 4 to 5          | Add SLG fragment | NcoI-BamHI (pSLG-test) | NcoI-BamHI |
| 5 to 6          | Add phiC31neo fragment | NheI-AvrII (phiC31neo insP4ins3-phiC31neo) | AvrII |
| 6 to 7          | Transfer to PAC vector | AscI-AvrII (arm-SLG-insP4ins3-phiC31neo) | AscI-AvrII |
| 8               | Linearization | PmeI |                         |
| 9               | Retrieving of BAC |             |                         |

BAC, bacterial artificial chromosome; HS4, DNase I hypersensitive site 4; PAC, P1 artificial chromosome; SLG, green-emitting luciferase.

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**A**

Genomic map of the mouse Hprt gene. Vertical black lines indicate exons. Numbers beside white boxes represent nucleotide positions of Hprt retrieving arms.

**B**

MI-MAC integration and the retrieved Hprt-SLG PAC vector. The MI platform consists of five phosphoglycerate kinase (PGK) promoter-attP arrays. FRT, phiC31attP, R4attP, TP901attP and Bxb1attP indicate yeast recombinase FLP, phage integrase phiC31, R4, TP901 and Bxb1 recognition sequence, respectively. Recombination between phiC31attB and attP by phiC31 integrase is represented.

**C**

Map of PAC vector integrated into the MI-MAC at the phiC31 site. Double-headed arrows with fragment length indicate regions amplified by PCR. Primer sequences are described in Table 2. Names of PCR-amplified regions are indicated to the left of the double-headed arrows.

Hprt, hypoxanthine guanine phosphoribosyltransferase; HS4, DNase I hypersensitive site 4; MI-MAC, multiple integration site-containing mouse artificial chromosome; PAC, P1 artificial chromosome; PGK, phosphoglycerate kinase; SLG, green-emitting luciferase.
DISCUSSION

In this report, we used BAC recombination and mammalian artificial chromosome technology to engineer an Hprt-reporter whose activity reflected living/dead cell viability in response to cytotoxic compounds.

Although the house-keeping Hprt gene has been used and verified as a standard for gene expression experiments, few reports have analyzed the Hprt gene promoter. Therefore, we examined a 20 kb long region of the Hprt promoter with the expectation of achieving reliable expression. Without using PCR cloning or a standard “cut and ligation” approach, we employed a BAC recombination method to acquire the long promoter region of the Hprt gene. In this Hprt promoter retrieving experiment, the BAC recombination efficiency (successfully retrieved bacterial clones) was 1%–6%. Retrieving rates we achieved for other genes were similar (data not shown). Recombination efficiency depends on the retrieving sequence and its length, and we considered the targeted Hprt promoter region within the limits of the procedure to achieve vector construction. Using a long Hprt promoter, we constructed the phiC31neoHprt-SLG reporter vector for integration at the phiC31 site on the MI-MAC. The phiC31neoHprt-SLG and phiC31 integrase expression vectors were co-transfected into A9 MI-MAC cells, and we isolated G418 resistant colonies. Transfected A9 MI-MAC clones were mostly positive for the 5′-junction PCR assay, but we detected only three lines with full-length integration (#2, #17 and #30; Table 3). These three lines produced nearly equivalent luciferase activity [clone #21 displayed low activity in spite of containing the SLG element (data not shown)]. We presume that low luciferase activity of clone #21 resulted from deletion of the Hprt promoter region during the integration step into the MI-MAC. While we employed a 39 kb PAC vector in this study, it is considered that intact integrations will occur at lower frequencies with larger vector constructions.

Luciferase activity of the three correctly integrated cell lines exhibited linearity with respect to cell numbers. We confirmed that this correlation was retained under continuous growth conditions for up to 72 h (Fig. 4A lower panel). We also confirmed proportionate decreases in luciferase activity and cell numbers under the toxic conditions of exposure to blasticidin or cisplatin (Fig. 4B). Blasticidin is a nucleoside antibiotic generally used in mammalian or bacterial cell selection and cisplatin is an anti-neoplastic drug widely used as a chemotherapeutic drug for cancer patients. Considering these results, the Hprt-SLG reporter can report on not only living (growing) cells, but also on toxicity-induced cell death. Although, cisplatin evoked a mild cytotoxic response up to 20 μM, we confirmed similar tendencies of luciferase activity and cell viability in response to blasticidin and cisplatin, suggesting that the Hprt-SLG reporter has the potential to evaluate cytotoxicity caused by different actions. We also note that employing a 20 kb Hprt promoter contributed to reliable expression of the Hprt-reporter gene on the MAC. Now, we are verifying the usability of the Hprt-SLG reporter in other cell lines by MAC transfer (manuscript in preparation).

Stability and uniformity of reporter gene expression are important factors and are required for reliable results in gene expression analyses. In addition, the transferrable feature of HACs and MACs by the microcell-mediated chromosome transfer method makes it possible to establish various cell lines using different recipients, including mouse embryonic stem cells. Transchromosomic mice generated from such HAC/MAC-transferred mouse embryonic stem cells can pave the way for authentic in vivo analysis. Recently, a simultaneous gene-loading system for HACs has been developed and is a further multi-purpose tool for gene analysis. Furthermore, an evaluation system for osteogenic differ-

| Primer name | 5′-sequence | Usage          |
|------------|-------------|----------------|
| PGK5       | AATGGAATGACGTCTCCTCCTACTAGTCTC | 5′-junction/long PCR |
| G418 3AS   | GGATGCACCGCTATGTCCCTGATAGCGGTC | 5′-junction PCR     |
| phiC31attL-B Fw2 | CTCGTCGGCGCTTGTGACG | 3′-junction PCR     |
| R4attP Rv  | AGTTGGGTGCAACCCCGAGAGTGTA | 3′-junction/long PCR |
| PAC#17     | CTCTAGCGGGGGATCTGCACTGCAC | Long PCR            |
| HPRT#31    | GTGTATAGGCGCCCTCTGTGCTGTTAAACCTG | Long PCR            |
| HPRT#33    | GTTACTATCGAGCTCGTGAACCCACGTGG | Long PCR            |
| HPRT#36    | CTCAGGCCCAGGTGGTAAAGCTCTGTC  | Long PCR            |
| HPRT#40    | GCGAGTGATTATCTGGAATCCCTCGGG  | Long PCR            |

HPRT, hypoxanthine guanine phosphoribosyltransferase; PAC, P1 artificial chromosome; PGK, phosphoglycerate kinase.
Table 3. Summary of PCR analysis of phiC31neoHprt-SLG vector-transfected MI-MAC A9 cells

| #Clone | JP-5 | JP-3 | LP-6 | LP-15 | LP-10 | LP-14 |
|--------|------|------|------|-------|-------|-------|
| 1      | –    | –    | +    | +     | +     | +     |
| 2      | +    | +    | +    | –     | +     | +     |
| 3      | +    | NT   | –    | –     | +     | +     |
| 4      | +    | NT   | +    | –     | +     | +     |
| 5      | +    | –    | –    | –     | –     | +     |
| 6      | +    | –    | +    | +     | +     | +     |
| 7      | +    | NT   | +    | –     | +     | +     |
| 8      | +    | NT   | –    | +     | +     | +     |
| 9      | –    | –    | –    | –     | +     | +     |
| 10     | +    | NT   | –    | –     | +     | +     |
| 11     | +    | –    | –    | –     | +     | +     |
| 12     | –    | –    | –    | –     | +     | +     |
| 13     | +    | –    | –    | –     | +     | +     |
| 14     | +    | NT   | +    | –     | +     | +     |
| 15     | +    | –    | +    | –     | +     | +     |
| 16     | –    | –    | +    | +     | +     | +     |
| 17     | +    | NT   | –    | –     | +     | +     |
| 18     | –    | –    | –    | –     | +     | +     |
| 19     | +    | –    | –    | –     | +     | +     |
| 20     | +    | NT   | +    | –     | +     | +     |
| 21     | +    | +    | –    | –     | +     | +     |
| 22     | +    | +    | –    | –     | +     | +     |
| 23     | +    | NT   | –    | –     | +     | +     |
| 24     | –    | –    | +    | +     | +     | +     |
| 25     | +    | NT   | +    | –     | +     | +     |
| 26     | +    | +    | –    | –     | +     | +     |
| 27     | +    | +    | –    | –     | +     | +     |
| 28     | +    | –    | –    | –     | +     | +     |
| 29     | +    | NT   | +    | –     | +     | +     |
| 30     | +    | +    | –    | –     | +     | +     |
| 31     | +    | –    | –    | –     | +     | +     |
| 32     | –    | –    | –    | –     | +     | +     |

Hprt, hypoxanthine guanine phosphoribosyltransferase; MI-MAC, multiple integration site-containing mouse artificial chromosome; NT, not tested; SLG, green-emitting luciferase.

Fig. 3. Luciferase activities and FISH analyses of Hprt-SLG cells.
A) Luciferase activities of A9 Hprt-SLG cells. Each clone was seeded 24 h prior to assay. Error bars represent standard deviation (n = 4).
B) Representative FISH image of A9 Hprt-SLG cells. Digoxigenin-labeled mouse minor satellite (red signal) and biotin-labeled Hprt-SLG PAC vector (green signal) were used as detection probes. Arrow indicates MI-MAC and insert shows magnified image of Hprt-SLG and MI-MAC signal. Hprt, hypoxanthine guanine phosphoribosyltransferase; MI-MAC, multiple integration site-containing mouse artificial chromosome; PAC, P1 artificial chromosome; SLG, green-emitting luciferase.
entiation has been established that utilizes a luciferase reporter and a MAC. The combination of multiple gene reporters, multiple gene loading and artificial chromosomes, will lead to diverse analyses of gene function and to high throughput systems that will contribute to drug development.

Noguchi et al. developed a dual-color luciferase assay system in which the expression of multiple genes can be tracked simultaneously using green- and red-emitting luciferases and this dual-color luciferase assay system was used for an in vitro test to screen skin sensitizer. By using green- and red-emitting luciferases as the internal control reporter and cytotoxicity specific reporter respectively, we can analysis quantitatively the cytotoxicity of chemical compounds.

In the future, I would like to develop an in-vitro nephrotoxicity test by using the dual-color luciferase assay system of Hprt-SLG reporter and nephrotoxicity marker gene- red-emitting luciferases.

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REFERENCES

1 Rubie C, Kempf K, Hans J, Su T, Tilton B, Georg T, et al. Housekeeping gene variability in normal and cancerous colorectal, pancreatic, esophageal, gastric and hepatic tissues. Mol Cell Probes. 2005;19:101-9. PMID: 15680211.
2 Frericks M, Esser C. A toolbox of novel murine house-keeping genes identified by meta-analysis of large scale gene expression profiles. Biochim Biophys Acta. 2008;1779:830-37. PMID: 18790095.
3 Svingen T, Letting H, Hadrup N, Hass U, Vinggaard AM. Se-
lecion of reference genes for quantitative RT-PCR (RT-qPCR) analysis of rat tissues under physiological and toxicological conditions. PeerJ. 2015;3:e855. PMID: 25825680.

4 Igarashi Y, Nakatsu N, Yamashita T, Ono A, Ohno Y, Urushidani T, et al. Open TG-GATEs: a large-scale toxicogenomics database. Nucleic Acids Res. 2015;43:921-7. PMID: 25313160.

5 Melton DW, McEwan C, McKie AB, Reid AM. Expression of the mouse HPRT gene: Deletional analysis of the promoter region of an X-Chromosome linked housekeeping gene. Cell. 1986;44:319-28. PMID: 3455894.

6 Magin TM, McEwan C, Milne M, Pow AM, Selfridge J, Melton DW. A position- and orientation-dependent element in the first intron is required for expression of the mouse hprt gene in embryonic stem cells. Gene. 1992;122:289-96. PMID: 1487143.

7 Viviani VR, Ohmiya Y. Bioluminescence color determinants of Phrixothrix railroad-worm luciferases: chimeric luciferases, site-directed mutagenesis of Arg 215 and guanidine effect. Photochem Photobiol. 2000;72:267-71. PMID: 10946582.

8 Kazuki Y, Oshimura M. Human artificial chromosomes for gene delivery and the development of animal models. Mol Ther. 2011;19:1591-601. PMID: 21750534.

9 Takiguchi M, Kazuki Y, Hiramatsu K, Abe S, Iida Y, Takehara S, et al. A novel and stable mouse artificial chromosome vector. ACS Synth Biol. 2014;3:903-14. PMID: 23654256.

10 Oshimura M, Uno N, Kazuki Y. A pathway from chromosome transfer to engineering resulting in human and mouse artificial chromosomes for a variety of applications to bio-medical challenges. Chromosome Res. 2015;23:111-33. PMID: 25657031.

11 Tomizuka K, Yoshida H, Uejima H, Kugoh H, Sato K, Ohguma A, et al. Functional expression and germline transmission of a human chromosome fragment in chimaeric mice. Nat Genet. 1997;16:133-43. PMID: 9171824.

12 Kazuki Y, Hiratsuka M, Takiguchi M, Osaki M, Kajitani N, Hoshiya H, et al. Complete Genetic Correction of iPS Cells From Duchenne Muscular Dystrophy. Mol Ther. 2010;18:386-93. PMID: 19997091.

13 Uno N, Uno K, Komoto S, Suzuki T, Hiratsuka M, Osaki M, et al. Development of a Safeguard System Using an Episomal Mammalian Artificial Chromosome for Gene and Cell Therapy. Mol Ther Nucleic Acids. 2015;4:e272. PMID: 26670279.

14 Kugoh H, Mitsuya K, Meguro M, Shigenami K, Schulz TC, Oshimura M. Mouse A9 cells containing single human chromosomes for analysis of genomic imprinting. DNA Res. 1999;6:165-72. PMID: 10470847.

15 Copeland NG, Jenkins NA, Court DL. Recombineering: a powerful new tool for mouse functional genomics. Nat Rev Genet. 2001;2:769-79. PMID: 11584293.

16 Lee EC, Yu D, Martinez de Velasco J, Tessarollo L, Swing DA, Court DL, et al. A highly efficient Escherichia coli-based chromosome engineering system adapted for recombinogenic targeting and subcloning of BAC DNA. Genomics. 2001;73:56-65. PMID: 11352566.

17 Yamada H, Li YC, Nishikawa M, Oshimura M, Inoue T. Introduction of a CD40L genomic fragment via a human artificial chromosome vector permits cell-type-specific gene expression and induces immunoglobulin secretion. J Hum Genet. 2008;53:447-53. PMID: 18322642.

18 Groth AC, Calos MP. Phage Integrase: Biology and Applications. J Mol Biol. 2004;335:667-78. PMID: 14687564.

19 Keravela A, Groth AC, Jarrahan S, Thayagarajan B, Hoyt II, Kirby PJ, et al. A diversity of serine phage integrases mediate site-specific recombination in mammalian cells. Mol Genet Genomics. 2006;276:135-46. PMID: 16699779.

20 Thayagarajan B, Olivares EC, Hollis RP, Ginsburg DS, Calos MP. Site-Specific Genomic Integration in Mammalian Cells Mediated by Phage phiC31 Integrase. Mol Cell Biol. 2001;21:3926-34. PMID: 11359900.

21 Olivares EC, Hollis RP, Calos MP. Phage R4 integrase mediates site-specific integration in human cells. Gene. 2001;278:167-76. PMID: 11707334.

22 Stoll SM, Ginsburg DS, Calos MP. Phage TP901-1 Site-Specific Integrase Functions in Human Cells. J Bacteriol. 2002;184:3657-63. PMID: 12057961.

23 Russell JP, Chang DW, Tretiakova A, Padidam M. Phage Bxb1 integrase mediates highly efficient site-specific recombination in mammalian cells. Biotechniques. 2006;40:460-4. PMID: 16629393.

24 Tasic B, Hippenmeyer S, Wang C, Gamboa M, Zong H, Chen-Tsai Y, Luo L. Site-specific integrase-mediated transgenesis in mice via pronuclear injection. Proc Natl Acad of Sci U S A. 2011;108:7902-7. PMID: 21464299.

25 Yamaguchi S, Kazuki Y, Nakayama Y, Nanba E, Oshimura M, Ohbayashi T. A method for producing transgenic cells using a multi-integrase system on a human artificial chromosome vector. PloS One. 2011;6:e17267. PMID: 21390305.

26 Yoshimura Y, Nakamura K, Endo T, Kajitani N, Kazuki K, Kazuki Y, et al. Mouse embryonic stem cells with a multi-integrase mouse artificial chromosome for transchromosomic mouse generation. Transgenic Res. 2015;24:717-27. PMID: 26055730.

27 Hamid R, Rotshteyn Y, Rabadi L, Parikh R, Bullock P. Comparison of alamar blue and MTT assays for high through-put screening. Toxicol In Vitro. 2004;18:703-10. PMID: 15251189.

28 Suzuki T, Kazuki Y, Oshimura M, Hara T. A novel system for simultaneous or sequential integration of multiple gene-loading vectors into a defined site of a human artificial chromosome. PloS One. 2014;9:e110404. PMID: 25303219.

29 Nara T, Katoh M, Inoue T, Taniguchi M, Kazuki K, Kazuki Y, et al. Construction of a luciferase reporter system to monitor osteogenic differentiation of mesenchymal stem cells by using a mammalian artificial chromosome vector. Yonago Acta Med. 2015;58:23-9. PMID: 26190894.

30 Noguchi T, Ikeda M, Ohmiya Y, Nakajima Y. A dual-color luciferase assay system reveals circadian resetting of cultured fibroblasts by co-cultured adrenal glands. PLoS One. 2012;7:e37093. PMID: 22615906.

31 Kimura Y, Fujimura C, Ito Y, Takahashi T, Nakajima Y, Ohmiya Y, et al. Optimization of the IL-8 luc assay as an in vitro test for skin sensitization. Toxicol In Vitro. 2015;29:1816-30. PMID: 26187477.