Chapter 28

Quantification of Genomic Mutations in Murine Hematopoietic Cells

Hartmut Geiger, David Schleimer, Kalpana J. Nattamai, and Jan Vijg

Summary

Maintaining the stability of the genome is critical to cell survival and normal cell growth. Genetic modification of hematopoietic cells might bear an inherent increased risk for the accumulation of DNA mutations. It frequently requires cultivation of the cells under super-physiological oxygen levels, which can result in increased oxidative damage, as well as under super-physiological concentrations of cytokines, which might interfere with DNA-damage checkpoint activation and by this means might result in an increased mutational load. We describe here a protocol for monitoring the frequency of DNA mutations in bone marrow cells post transduction or upon selection either in vitro or in vivo based on the lacZ-plasmid (pUR288) transgenic mouse (small blue mouse) mutation indicator strain.

Key words: Hematopoietic cells, Gene transfer, Mutation, DNA damage, Transgenic, pUR288.

1. Introduction

The lacZ-plasmid (pUR288) transgenic mouse (small blue mouse) has been generated to serve as an in vivo indicator for mutagenic events in the somatic genome *(1)*. In this model, nontranslated concatameric plasmids that are integrated into the murine genome can be analyzed for mutations in the lacZ part of the plasmid. For the quantification of somatic mutations in this model, the plasmids are excised from the genomic DNA, enriched with magnetic beads, ligated and electroporated into a distinct *E. coli* strain that allows for metabolic selection of plasmids that bear mutations in the lacZ gene. The type of mutation is then determined by a PCR in combination with restriction digestion.

Christopher Baum (ed.), *Methods in Molecular Biology, Methods and Protocals, vol. 506*

DOI: 10.1007/978-1-59745-409_28

The use of this animal model for the quantitative determination of the frequency and nature of somatic mutations due to for example organismal aging in various tissues has been extensively characterized *(2–11)*. Here we present a protocol specifically adapted for lacZ mutation analysis in murine hematopoietic cells *(8)*.

2. Materials

2.1. Mice

C57BL/6-Tg(LacZpl)60Vij/J (little blue mice) can be obtained from The Jackson laboratory (http://jaxmice.jax.org), stock number 002754.

2.2. DNA Isolation

QIAamp Blood Midi Kit (Qiagen, Valencia, CA).

2.3. Competent Cells

1. LB medium: 5g tryptone (Difco, Sparks, MD), 2.5g yeast extract (Difco), 5g NaCl, 500mL water, autoclave for 20min (*see* **Notes 1–3**).
2. puC19 or pUR288 DNA is dissolved at 0.04 or 0.4pg/μL in water, store at –80°C at 5μL single-use aliquots.
3. TB medium: 0.9g tryptone, 1.18g yeast extract, 0.47g K_2HPO_4, 0.11g KH_2PO_4 is dissolved in 50mL water, autoclave for 20min.
4. Top agar: 1.0g tryptone, 0.5g yeast extract, 0.025g NaCl, 0.35g agar (Difco) is dissolved in 100mL water, autoclave for 20min, keep top agar at 41°C.

2.4. Preparation of lacZ/lacI Fusion Protein

1. Streak lacZ–LacI fusion protein producing strain on M9CA plates (Difco, add 0.35g agar/100mL), grow overnight at 30°C.
2. Select at least five colonies, inoculate in 1.5mL M9CA medium, streak 100μL of medium after 4h on TY X-gal plates (to 100mL TY medium (Difco) add 0.35g agar, autoclave for 20min, add X-gal (to 75μg/mL)), incubate the plates and the cultures overnight at 30°C.
3. Select the colony with the highest beta-galactosidase activity (bluest plate), inoculate overnight culture in 50mL M9CA medium, culture again overnight at 30°C.
4. Transfer 30mL of this culture to 8L of TY medium, incubate at 30°C to OD_{600} of 1.5 (late log phase), monitor growth every 30min after first 3h.
5. Weigh centrifuge beakers. Concentrate cells by centrifugation at 4,000×*g* for 20min.
6. Resuspend pellet carefully in 1mL 50mM Tris–HCl (pH 7.5), 10% (w/v) sucrose per 1g of cell pellet.

7. Drip cell slurry slowly into liquid nitrogen (will generate popcorn like structures), harvest popcorn and store at −80°C.
8. Dissolve two vials of protease inhibitor cocktail VI (A.G Scientific) in 75mL of 50mM Tris–HCl, pH 7.5/10% (w/v) sucrose and prewarm to 37°C. Take 60g of frozen cell slurry and add it quickly to this solution. Stir carefully, add 7.5mL lysis solution (2M NaCl, 7.6% (w/v) spermidine·HCl, 10% (w/v) sucrose), add 30mg lysozyme, mix carefully, and leave on ice for 1h.
9. Transfer the suspension to a 37°C water bath for 4min, swirl every 30s, then put suspension back on ice.
10. Centrifuge for 1h at 23,000 × *g* at 4°C. Pour supernatant off, measure volume, and put it on ice.
11. While stirring, add ammonium sulfate to the supernatant to 40% saturation (24 g/100 mL supernatant), stir on ice for another 2 h.
12. Centrifuge at 18,000×*g* for 15min at 4°C. Discard supernatant.
13. Resuspend pellet in 6mL of storage buffer (25mM Tris–HCl (pH 8.0), 150mM NaCl, 1mM EDTA, 5mM $MgCl_2$, 0.05% (v/v) Tween 20, 25% (v/v) glycerol, 14.3mM beta-mercaptoethanol), store at single-use aliquots (10μL) at −80°C.

2.5. Preparation of lacI/lacZ Coated Magnetic Beads

1. Take 1mL of Dynal magnetic beads coated with sheep anti-mouse IgG (4×10^8 magnetic beads/mL) and pellet the beads on a magnetic stand. Wash the beads 3 times in 1mL PBS.
2. Resuspend the magnetic beads in 950μL PBS and 50μL anti-beta-galactosidase antibody (2mg/mL, Promega, Madison, WI)). Incubate at 37°C for 1h while rotating. Wash the beads 3 times in 1mL PBS as earlier.
3. Resuspend beads in 990μL of PBS and 10μL of lacZ/lacI fusion protein. Incubate at 37°C for 2h while rotating. Wash the beads 3 times in 1mL PBS as earlier. Resuspend the beads in 1mL PBS and store at 4°C. Coated beads are good for at least 6 months.

2.6. Plasmid Enrichment and Electroporation Procedure

1. 5× binding buffer: 50mM Tris–HCl (pH 7.5), 50mM $MgCl_2$, 25% (w/v) glycerol, adjust to pH 6.8 with HCl, sterilize with 0.22-μm filter. Store at room temperature. To obtain 1× buffer, dilute with water.
2. IPTG stock solution: dissolve IPTG (isopropyl-beta-d-thiogalactopyranoside) at 25mg/mL in water, sterilize with 0.22-μm filter, store in single-use aliquots (60μL) at −20°C.
3. IPTG elution buffer: 10mM Tris–HCl (pH 7.5), 1mM EDTA, 125mM NaCl, sterilize with 0.22-μm filter, store at room temperature.
4. ATP: dissolve at 10mM in water, sterilize with 0.22-μm filter, store in single-use aliquots (10μL) at −80°C.

5. 3M Sodium acetate (pH 4.9), sterilize with 0.22-µm filter.
6. X-gal stock solution: dissolve 50mg X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) in dimethylformamide, store protected from light at 4°C.
7. TB/glycerol medium: 0.9g tryptone, 1.18g yeast extract, 0.47g K_2HPO_4, 0.11g KH_2PO_4 is dissolved in 50mL water, autoclave for 20min. Add 400µL of 50% (w/v) glycerol to the medium and mix. Prepare always fresh.
8. Top-agar: 1.0g tryptone, 0.5g yeast extract, 0.025g NaCl, 0.35g agar is dissolved in 100mL dH_2O, autoclave for 20min, keep top agar at 41°C. Prepare 2 times 100mL. To prepare X-gal top agar, add to 100mL top agar 50µg/mL 2,3,5-triphenyl-2H-tetrazolium chloride, 25µg/mL kanamycin, 150µg/mL ampicillin and 37.5µg/mL X-gal. To prepare p-gal top agar, add to 100mL top agar 50µg/mL 2,3,5-triphenyl-2H-tetrazolium chloride, 25µg/mL kanamycin, 150µg/mL ampicillin and 0.3% (w/v) p-gal (phenyl-beta-D-galactopyranoside).

2.7. Analysis of Mutant Clones

1. LB medium (*see* **Subheading 2.3**).
2. X-gal plates for galactose-insensitive screening: to 500mL water, add 20 capsules LB Agar, autoclave for 20min. Then add kanamycin (to 25µg/mL), Ampicillin (to 150µg/mL), X-gal (to 75µg/mL), pour 35mL agar per 15-cm plate and dry in dark. Store plates at 4°C.
3. PCR Master mix (per sample): 11.5µL water, 0.5µL 12.5µM pUR4923-F(5′-TGG AGC GAA CGA CCT ACA CCG AAC TGA GAT-3′), binds to the ORI of pUR288, 0.5µL 12.5µM pUR3829-R (5′-ATA GTG TAT GCG ACC GAG TTG CTC TTG-3′), binds to the Ampicillin resistance gene locus in pUR288, 12.5µL Qiagen HotStarTaq Master Mix.
4. PCR program pUR288CO: Step 1: 95°C, 10min; Step 2: 95°C, 20s; Step 3: 68°C, 8min; Step 4: go to step 2 for 34 more times; Step 5: 68°C, 10min; Step 6: 4°C, ∞.
5. 1% TBE gel: 1% agarose (w/v) in 1× TBE-buffer, add 2µL ethidium bromide solution per 100mL gel (from stock at 10mg/mL).
6. 6× Gel loading buffer: 0.25% (w/v) Bromophenol Blue, 0.25% (w/v) xylene cyanol FF, 30% glycerol (w/v).

3. Methods

There are now multiple types of transgenic mice available for mutagenicity testing as well as mice that utilize endogenous reporter genes as readout targets. The mutation assay based on

the pUR288 transgenic plasmid systems allows qualitative as well as quantitative comparisons of the mutation frequency in almost any given tissue/cell type in vivo as well as in vitro, as it does not, as many other models, require in vitro proliferation/selection of primary single cell clones (like for example the HPRT or APRT mutation indicator systems). The pUR288 system detects point mutations and other small intragenic lesions as well as large deletion and nonhomologous recombination events. However, it cannot detect other large chromosomal events such as mitotic recombination (LOH) or nondysjunction events. Data generated in our laboratory indicate that with a sample size of ten biological repeats, the assay has the power to assign a twofold difference in the total mutation frequency among experimental groups with statistical significance of $p<0.05$.

3.1. Preparation of Electrocompetent Cells

1. Prepare 1L sterile ice-cold water and 125mL sterile ice-cold 10% glycerol in water.
2. Add 50µL of an *E. coli* C (ΔlacZ/galE$^-$) glycerol stock and 5µL of 50mg/mL kanamycin to 10mL LB medium. Incubate overnight at 37°C at 250rpm. We always start two cultures, in case one does not grow.
3. Next morning: Make sure the rotor for the 50-mL round-bottom tubes is refrigerated.
4. Resuspend the overnight cultures carefully, add 1.5mL of the overnight culture to each of two Erlenmeyers with 500-mL LB medium which contains no kanamycin (*see* **Notes 2** and **3**). Grow the cells to an OD_{600} of 0.45 at 31.5°C and 250 rpm. Take the cell density every 15min after the first 3h. Cells should follow the growth curves depicted in **Fig. 1a** and **Fig. 1b**.Discard flask that do not follow these curves (e.g., cells growing faster or slower).
5. Cool the cells by placing the Erlenmeyers on ice for 30 min on a shaker. Use styrofoam boxes filled with ice to hold Erlenmeyers as you will work always on ice from now on.
6. Divide the cell cultures over two 500-mL centrifuge bottles, centrifuge the cells at 3,800 × *g* for 15min at 4°C.
7. Very gently resuspend each pellet in 250mL ice-cold water, shake slowly on ice on rotary shaker until pellet is dissolved. Never pour water over pellet. Centrifuge the cells again at 3,800 × *g* for 15min at 4°C. Take the supernatant off by vacuum, but never touch the pellet directly.
8. Repeat **step** 7.
9. Resuspend the cell pellets in 50mL 10% (w/v) ice-cold glycerol (in water) by shaking tubes on ice on rotary shaker and divide the two suspensions over four 50-mL round-bottom high-speed tubes. Centrifuge cells at 3,800 × *g* for 15min at 4°C. Take the supernatant off by vacuum.

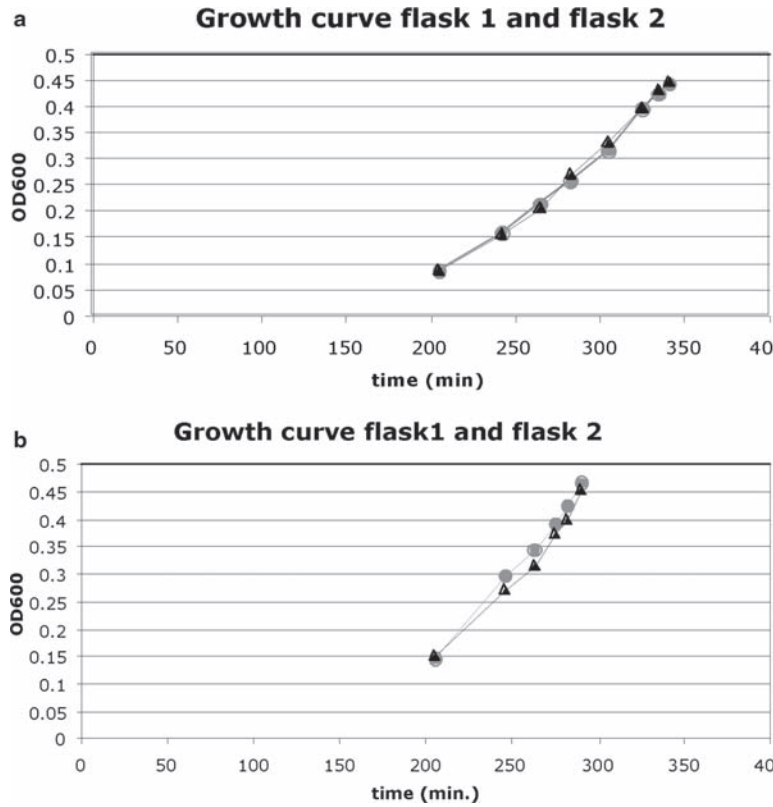

Fig. 1. (**a** and **b**) Representative growth curves for E. coli C (ΔlacZ/galE⁻) strains for the preparation of electrocompetent cells from two independent experiments.

10. Resuspend each pellet in 0.5mL 10% (w/v) ice-cold glycerol (shake on ice on rotary shaker) and combine the pellets. The OD_{600} should be the following: 10μL cells in 3mL LB should have an OD_{600} of at least 0.34 up to 0.41; adjust density of the suspension if necessary with 10% (w/v) glycerol (linear math if density is too high). Keep cells on ice.
11. Aliquot the cell suspension into microcentrifuge tubes at 255μL per tube and freeze tubes immediately in a dry ice/ethanol bath. Add number of vials generated, date of production, and the information on growth curve into the database. Store cells at −80°C (*see* **Note 4**).
12. Test competency of the cells the next day: Take 5μL of pUR288 at 0.4pg/μL at a total of 2pg (or a total of 0.2pg pUC19) and electroporate competent cells according to a standard protocol (*see* **Subheading 2.6**), and plate on X-gal top agar (*see* **Subheading 2.6**). Count colonies the next day. Be sure to always include as a control electrocompetent cells from a previous cell preparation with a known efficiency.

13. Calculate the efficiency:

$$\frac{\text{Number of colonies}}{2\,(\text{pg})\text{ of DNA}} \times \frac{1\times10^{6}\ \text{pg}}{\mu\text{g}} \times 100(\text{dilutionfactor})$$

14. We expect to obtain at least around 1.5×10^{10} colonies/μg plasmid when cells are tested with pUC19 and tenfold less when tested with pUR288.

3.2. DNA Purification from Bone Marrow Cells

1. Bone marrow cells are flushed out of the femur and/or tibia with a syringe and a 20–22G needle in a volume of at least 2mL of PBS. Take the cell count, and aliquot at least 1×10^{7}cells into a microcentrifuge tube (make aliquots in case there are more than 1×10^{7}cells).
2. Cells will be centrifuged at $1{,}500\times g$ at 4°C for 5min. The supernatant will be discarded and the cells resuspended in 1.5mL of PBS. Cells will be again centrifuged at $1{,}500\times g$ at 4°C for 5min and the supernatant will be discarded. Quick freeze cell pellet in ethanol/dry ice bath. Store cell pellet at −80°C for further use.
3. At least 1×10^{7}cells should be used for the DNA isolation to obtain enough DNA for the assay. Thaw cell pellet and follow precisely instructions provided with the QIAamp Blood Midi Kit.

3.3. Determination of the Frequency of Mutated Plasmids via the Plasmid Enrichment and Electroporation Procedure

1. Adjust 10–30μg genomic DNA to a 150-μL volume with water (we always use 30μg in case the samples were heterozygous for the lacZ transgene). Add 30μL of 5× binding buffer and 60U of HindIII. Mix gently and incubate mixture for 1h at 37°C in water bath. The time can be extended to up to 2h. As a control, always perform the assay on DNA from a non-pUR288 transgenic tissue at the same time (*see* **Note 5**).
2. Pellet 60μL of lacZ/lacI fusion protein-coated beads using the magnetic stand and remove the PBS storage buffer. Resuspend the beads in the DNA/HindIII/binding buffer mixture. Incubate for 1h at 37°C while rotating.
3. Wash the beads 3 times with 250μL 1× binding buffer and resuspend them in 75μL IPTG-elution buffer, 5μL IPTG stock solution, 100μL water, and 20μL NEBuffer #2. Vortex softly and incubate for 30min at 37°C while rotating.
4. To further support dissociation of the plasmid from the beads as well as inhibition of the restriction enzyme, place mixture at 65°C for 20min. Allow cooling to room temperature and spin shortly to get down condensation drops. Add freshly thawed ATP to a final concentration of 0.1mM (2μL of a

10mM stock) and 0.1U T4 DNA ligase (1µL of a 10× dilution of 1U/µL T4 DNA ligase in 1× T4 DNA ligase buffer). Vortex softly and incubate at room temperature for 1h.

5. Resuspend beads by light vortexing (for better suspension) and pellet them on the magnetic stand and transfer the solution (without beads) to a clean tube. Repeat this two more times to remove all remaining beads in the solution.
6. Precipitate DNA with 30µg glycogen, 0.1volume 3M sodium acetate, pH 4.9 (approximately 22µL) and mix before ethanol is added. Add 2.5volumes of 95% ethanol (approximately 560µL). Vortex and place mixture at −80°C for at least 30min for precipitation. A southern blot tracking the plasmid along these steps is depicted in **Fig. 2** (excision from genomic DNA, enrichment, ligation, precipitation).
7. While the DNA is precipitating at −80°C, prepare X-gal and p-gal top agar.
8. Spin down the precipitated DNA for 30min at 14,000×*g* at 4°C. Wash the pellet once with 250µL 70% (v/v) ethanol, vortex, and spin again at 14,000× *g* for 5min. Carefully remove all remaining ethanol using a fine tip pipette and allow the DNA to dry for at least 10min at room temperature. Resuspend the DNA in 5µL water.
9. While the DNA is drying, place electroporation cuvettes (0.1-cm gap width) and electrocompetent cells (directly out of −80°C) on ice for a minimum of 20min.
10. Set Gene Pulser to 25mF and 1.8kV. Set Pulse Controller to 200W (*see* **Note 6**).

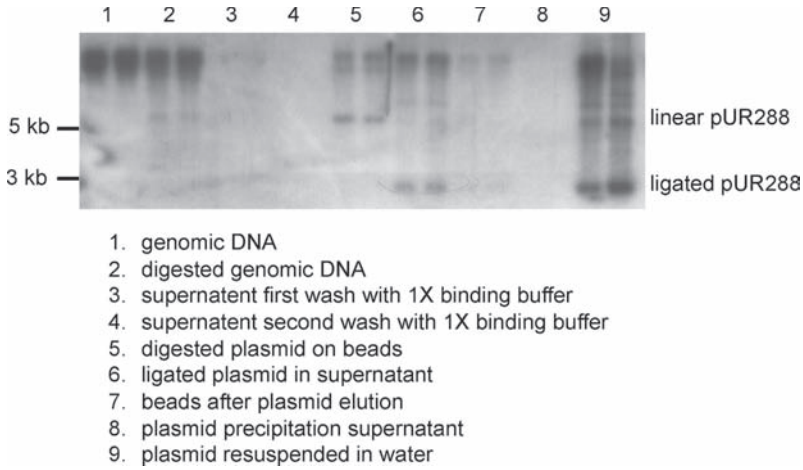

Fig. 2. Southern blot depicting the whereabouts of the plasmid pUR288 along the enrichment procedure. The blot demonstrates that the plasmid is highly enriched in the final precipitated product by the procedure, with only minor plasmid loss in any of the supernatants analyzed.

11. Pipet 60μL of electrocompetent cell slurry (*E. coli* lacZ/galE$^-$) carefully to the 5μL plasmid solution. Mix cells with the plasmid carefully by swirling the mixture with pipette tip (do not pipet up and down). Pipet cells/plasmid mixture into electroporation cuvette and electroporate.
12. After the electroporation, immediately add 1mL of ice-cold TB medium to the cuvette.
13. Transfer the cells to a 15-mL culture tube containing an additional 1mL TB medium and incubate at 37°C for 1h at 225rpm in a shaker/incubator.
14. Dilute either 2 or 4μL of the cell suspension in 2mL TB medium which is mixed with 13mL X-gal top agar and plated. We target this dilution to obtain later at least 100 colonies on the X-gal plate. The remaining cells (1.998 or 1.995mL) are plated using 13mL p-gal top agar. Wait until top agar is solidified (usually takes 30min).
15. All plates are incubated for 15h at 37°C.
16. Count colonies on both p-gal and X-gal top agar plates. The raw mutation frequency is determined by dividing the total number of colonies on the selective plate (p-gal) by the number of colonies on the titer plate (X-gal) and the dilution factor (500× or 1,000×). The final mutation frequency will be determined after the analysis of the mutant clones.

3.4. Analysis of Mutant Clones

1. To determine the type of mutation of the clone, the following PCR/restriction digest procedure is performed. The PCR amplifies the part of the plasmid containing the lacZ gene.
2. Transfer individual mutant colonies from selective plates to individual wells of 96-well round-bottom-plates in an LB medium containing ampicillin (150μg/mL) and kanamycin (25μg/mL). Works best with 2-μL tips.
3. Cover and tape plate shut. Grow overnight in a shaker/incubator at 37°C and 200rpm.
4. Examine overnight cultures for growth. Note any cultures that did not grow on a spreadsheet (follow 12 by 8 matrix of the 96-well plate).
5. Transfer a sample of each culture with a Boekel replicator to an X-gal plate for galactose sensitivity screening. Mark the right orientation on the bottom plate and incubate upside down at 37°C. Examine plate after 1–2h for blue staining cultures; these are galactose-insensitive host cells containing wild-type plasmids (false mutants, *see* **Fig. 3**). Circle blue colonies and note them on spreadsheet.
6. Incubate plate overnight: Note any colonies that did not grow on spreadsheet (false mutants, *see* **Fig. 4**).

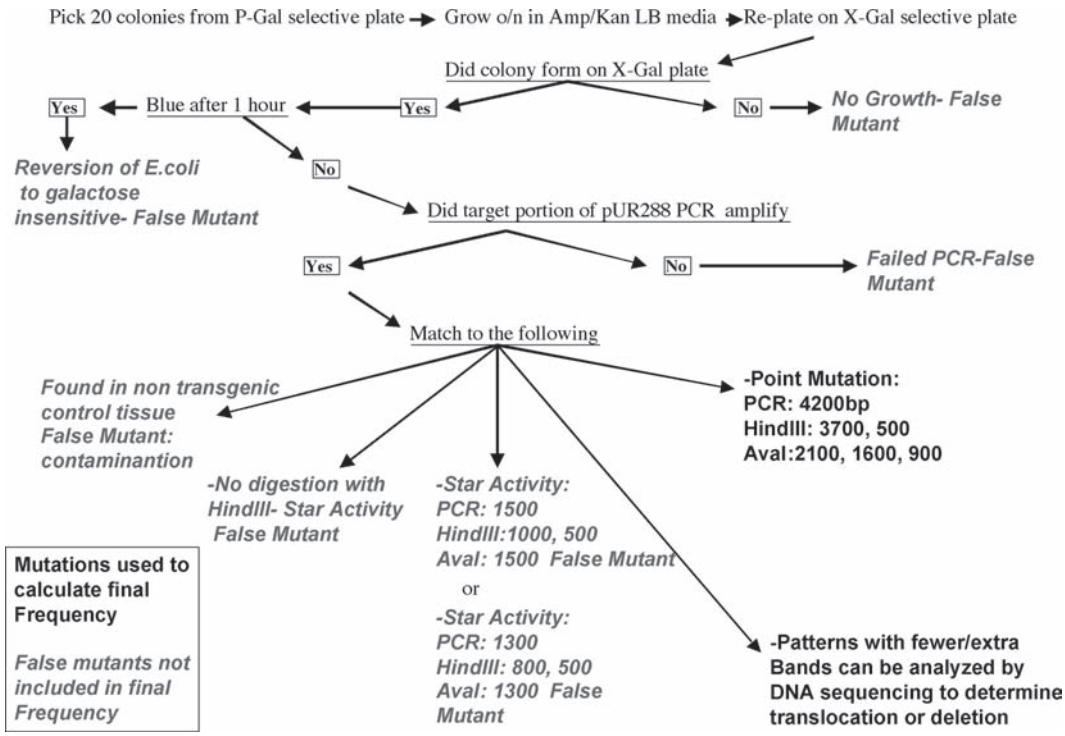

Fig. 3. Decision tree for determining the final mutation frequency from the raw mutation frequency in combination with the analysis of the mutant clones picked from the p-gal top agar plate. Any mutant clone that falls in a "black" category will be counted as a mutant clone and will be used to determine the final mutation frequency according to the formula in Subheading 3. Any clone that falls into a "lighter gray" area will be regarded as false positive and discarded. Up to 30% of all clones might fall into that group.

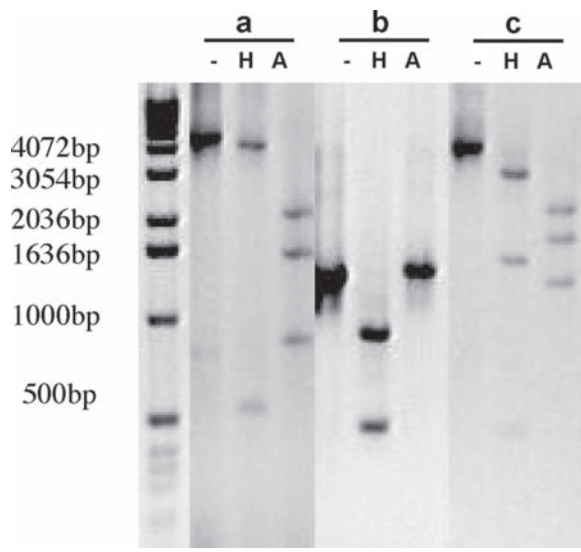

Fig. 4. Typical restriction patterns for mutant clones, depicting (**a**) a pattern resembling a point mutation, (**b**) a pattern resembling a star activity, and (**c**) a pattern resembling either a deletion or a translocation (size-change mutation). *H* digested by HindIII; *A* digested by AvaI.

7. Transfer 2μL of each culture with a multichannel pipetter to a 96-well plate containing the PCR Master mix. Run the PCR.
8. Prepare Mastermix for PCR product digestions as follows:

HindIII (per sample)	AvaI (per sample)
10.25μL water	10μL water
1.5μL NEBuffer# 2	1.5μL NEBuffer# 4
0.25μL HindIII (5U)	0.5μL AvaI (5U)

9. Load 12μL of each master mix into an individual well of a 96-well plate, add 3μL of the PCR product to the digestion master mix and incubate at 37°C for 1h.
10. Once digestion is complete, load samples onto a 1% TBE gel the following way: Aliquot 5μL of undigested PCR product, add 5μL 6× loading buffer. Mix and load 8μL to gel. Add 5μL 6× loading buffer directly to each digested sample and load 8μL to gel.
11. Samples should be loaded in the following order: PCR product undigested, HindIII digest, AvaI digest. Load 5μL 1-kb DNA size marker to flank each row of samples.
12. Run gel for 2h at 100V. Take gel picture.

3.5. Scoring of Mutant Clones

1. Depending on the length of the PCR product and the type of the pattern of the digested clones, three distinct types of mutations can be identified after gel electrophoresis (**Fig. 4**).
   - Nonsize-change mutations (point mutations). The PCR amplifies a 4.2-kb part of pUR288, containing the lacZ part of the gene. A clone that shows the size and restriction pattern identical to the PCR product generated from the parental pUR288 plasmid (undigested: 4.2kb; HindIII: 3.7, 0.5kb; AvaI: 2.1, 1.6, 0.9kb) is regarded to bear a point mutation that renders the lacZ gene inactive. Such clones are scored as point mutations (**Fig. 4**, pattern a).
   - Mutant clones due to star activity: Under certain extreme conditions, restriction endonucleases are capable of cleaving at sequences which are similar but not identical to their defined recognition sequence (star activity), which will usually not result in regeneration of the consensus HindIII restriction recognition site upon religation. So any PCR product that is not recut by HindIII is regarded to be such a product (which does not count as a mutation conferred upon in the animal and thus is regarded as a false mutant) and will not be scored and

thus subtracted from the number of mutant clones. Secondly, as confirmed by DNA sequencing, two of the star activity sites in PCR product will regenerate a HindIII restriction site upon religation, but these clones show a very characteristic gel pattern: The PCR product is either 1.5 or 1.3kb, while the HindIII sizes are 1.0 and 0.5 or 0.8 and 0.5kb and the product is uncut by AvaI (**Fig. 4**, pattern b). These clones will not be scored as mutations and thus subtracted from the number of mutant clones.

- Size-change mutations (deletions, translocations, and inversions). Any clone that deviates from the point mutation pattern as described in (a) and does not register as a star activity mutation will be scored as a mutation that is either a deletion or a translocation (**Fig. 3**, pattern c). A single sequencing reaction though can be used to identify translocations among these types of mutations (*see* **Note 8**).
- Contaminations. Any clone that is lacZ negative, but resistant to kanamycin and ampicillin will read out as a mutant clone in this assay. Thus even a very minor contamination with such a plasmid can result in false positive mutant clones in the p-gal plate. As we carry DNA from a nontransgenic animal alongside with test-DNA as a control in this assay, any mutant clone that is found in the nontransgenic sample is regarded as a contamination. This also means that any clone in a test sample with an identical restriction pattern has to be subtracted from the number of mutant clones in this sample (*see* **Note 8**).

2. Finally, any clone that did not amplify by PCR is regarded a false positive and will not be scored as a mutation.
3. In summary, this results in the "decision tree" diagram shown in **Fig. 4.** Follow that diagram to correctly subtract false mutants from the raw mutation frequency and to then determine the final mutation frequency of the sample with the formula given in **Subheading 3.1** and the percen-tage of point mutations and large deletions/translocations (*see* **Notes 7** and **8**).

4. Notes

1. We always use either ddH_2O or ultrapure 18.2MΩcm resistivity water when we refer to water.
2. We found that the source of the water used for making the electrocompetent cells does influence the efficiency for electroporation of the product. So it might be a good idea

to initially test water from different purification systems/resources in the facility.

3. Do not use pellets to prepare the LB for making electrocompetent cells; always prepare from powder and always prepare medium fresh.
4. Do not store electrocompetent cells longer than 3 months. They may lose competency.
5. As any lacZ-kan+amp+plasmid will read out as a mutation in this assay, it is central for the assay to always carry along a nontransgenic tissue throughout the analysis, as well as to have dedicated pipettes, reagents, and equipment that are not shared with any other kind of plasmid work (like minipreps for example).
6. The settings for the electroporation might have to be tested/optimized on your specific electroporator. We use a product from BioRad (Hercules, CA).
7. To unequivocally identify the type of mutation, the whole PCR product has to be sequenced. As in almost all instances though a point mutation pattern by restriction digestion was verified as a point mutation by sequencing, the current approach is a straightforward and relatively inexpensive but reliable way to determine the type of mutation.
8. If it is not clear whether the clones are contaminations, sequencing is a very straightforward way to identify whether the clone is derived from pUR288 (most likely mutation) or not (contamination). We use the following sequencing primer pUR3839.R (5′-ATA GTG TAT GCG GCG ACC GAG TTG CTC TTG-3′). The elongation of this primer spans the transition from the plasmid backbone over the HindIII restriction site into the lacZ part of pUR288. Also based on this one primer-sequencing reaction, it is possible to distinguish between deletions and translocations (plasmid contains sequences from murine genomic DNA, see examples in **Table 1**.

Table 1
Mutation frequency ($\times 10^{-5}$)

Bone marrow: 6.0±3.5			**Small intestine: 12.8±7.9**		
Point mutations	Translocations	Deletions	Point mutations	Translocations	Deletions
20%	59%	21%	61%	11%	28%

Bone marrow shows only half the mutational load in young animals compared to small intestine. Interestingly though, bone marrow has with 60% of all mutant clones being translocations the highest spontaneous rate of translocations for any tissue reported so far

Acknowledgments

The author would like to thank Martijn Dollé and Rita Busuttil for their support in learning how to perform the assay and Sina Albert for support in establishing the assay in his laboratory. This work was supported in part by NIH grant R01 HL076604 as well as a New Scholar in Aging grant from The Ellison Medical Foundation.

References

1. Dolle, M.E., et al., *Evaluation of a plasmid-based transgenic mouse model for detecting in vivo mutations.* Mutagenesis, 1996. **11**(1): 111–8.
2. Dolle, M.E., et al., *Rapid accumulation of genome rearrangements in liver but not in brain of old mice [see comments].* Nat Genet, 1997. **17**(4): 431–4.
3. Dolle, M.E., et al., *Distinct spectra of somatic mutations accumulated with age in mouse heart and small intestine.* Proc Natl Acad Sci U S A, 2000. **97**(15): 8403–8.
4. Giese, H., et al., *Age-related mutation accumulation at a lacZ reporter locus in normal and tumor tissues of Trp53-deficient mice.* Mutat Res, 2002. **514**(1–2): 153–63.
5. Vijg, J. and M.E. Dolle, *Large genome rearrangements as a primary cause of aging. Mech Ageing Dev,* 2002. **123**(8): 907–15.
6. Dolle, M.E. and J. Vijg, *Genome dynamics in aging mice.* Genome Res, 2002. **12**(11): 1732–8.
7. Tutt, A.N., et al., *Disruption of Brca2 increases the spontaneous mutation rate in vivo: synergism with ionizing radiation.* EMBO Rep, 2002. **3**(3): 255–60.
8. Geiger, H., et al., *Mutagenic potential of temozolomide in bone marrow cells in vivo.* Blood, 2006. **107**(7): 3010–1.
9. Vijg, J., et al., *Transgenic mouse models for studying mutations in vivo: applications in aging research [corrected and republished article originally printed in* Mech Ageing Dev, 1997. **98**(3): 189–202]. Mech Ageing Dev, 1997. **99**(3): 257–71.
10. Dolle, M.E., et al., *Characterization of color mutants in lacZ plasmid-based transgenic mice, as detected by positive selection.* Mutagenesis, 1999. **14**(3): 287–93.
11. Dolle, M.E., et al., *Background mutations and polymorphisms in lacZ-plasmid transgenic mice.* Environ Mol Mutagen, 1999. **34**(2–3): 112–20.