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An approach for controlling the timing and order of engineered mutations in mice

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Summary

Significant advances in our understanding of normal development and disease have been facilitated by engineered mice in which genes can be altered in a spatially, temporally, or cell type restricted manner using site specific recombinase systems like Cre-loxP or Flp-frt. In many circumstances it is important to understand how interactions between multiple genes influence a given phenotype. Robust approaches for precisely controlling multiple genetic alterations independently are limited, however, thus the impact of mutation order and timing on phenotype is generally unknown. Here we describe and validate a novel Gt(ROSA)26Sor targeted transgene allowing precise control over the order and timing of multiple genetic mutations in the mouse. The transgene expresses an optimized, Flp-estrogen receptor fusion protein (Flpo-ERT2) under the control of a loxP-stop-loxP cassette. In this system, genes modified by loxP sites are altered first upon expression of Cre. Cre also eliminates the loxP-stop-loxP cassette, permitting wide-spread expression of Flpo-ERT2. Because of the estrogen receptor fusion, Flp activity remains inert until administration of tamoxifen, allowing genes modified by frt sites to be modified subsequently with controllable timing. This mouse transgene will be useful in a wide variety of applications where independent control of different mutations in the mouse is desirable.

Keywords

cancer; development; mouse genome engineering; site specific recombinases

1 | INTRODUCTION

Genetically engineered mouse models (GEMM) have been invaluable for elucidating the function of genes in normal development and disease. An important advance in the utility of GEMMs is the ability to induce genomic changes in a temporally and spatially controlled manner, often through the use of site specific recombinase systems like Cre-loxP or Flp-frt (Fiering, Kim, Epner, & Groudine, 1993; Lakso et al., 1992; Orban, Chui, & Marth, 1992). By engineering recombination sites into the mouse genome, recombination events can be controlled by regulated expression of the recombinase. For example, by controlling Cre expression with tissue restricted promoter/enhancer elements, loxP recombination events creating activating or debilitating mutations can be restricted to a particular tissue. Temporal

control of recombinase expression can be accomplished using inducible promoters like tetracycline inducible systems (Furth et al., 1994) or through the use of estrogen receptor recombinase fusion proteins which block nuclear recombinase activity until the administration of the estrogen receptor ligand tamoxifen (Indra et al., 1999).

In many circumstances it is desirable to engineer mutations in multiple genes to assess genetic relationships pertinent to developmental or disease phenotypes under study. When multiple mutations are engineered into mice currently, the order and timing of those mutations is not typically controlled. For example, recombination sites (i.e., loxP) are often engineered into the mouse genome at several locations to alter different loci. Once the recombinase is expressed or activated, recombination is assumed to occur in all loxP modified loci at random over short periods of time such that gene deletion is considered effectively simultaneous. However, different loxP modified loci may recombine with different efficiencies. Thus recombination may favor a particular but undefined order based on their relative recombination efficiencies. Whether this order of mutation affects resulting phenotypes is not usually known. Further, since recombination may not proceed to completion within biologically relevant time frames, tissues may be mosaic with individual cells containing different combinations of recombined and unrecombined alleles. In such cases it is difficult to determine whether observed effects are cell autonomous. These potential effects may be particularly important in processes like development or cancer progression where the evolutionary trajectory of cells is likely influenced by the cell state or genetic background upon which new genetic alterations occur.

Here we describe a novel approach to address this limitation. We make use of both the Cre-loxP and Flp-*frt* recombination systems with a tamoxifen inducible Flp recombinase estrogen receptor fusion gene in a configuration that allows two different mutations, or sets of mutations, to be induced with defined order and timing. This approach will be useful in experiments where controlling the order of multiple genetic alterations at particular time points within a given cell lineage is desired.

2 | RESULTS AND DISCUSSION

We designed and constructed a vector to target the Gt(ROSA)26Sor allele (Friedrich & Soriano, 1991) with a transgene expressing an optimized Flp recombinase in a dual-inducible manner (Figure 1a). Transgene expression is driven by the strong, ubiquitous CAG promoter (Miyazaki et al., 1989), but proper translation of the RNA transcript is prevented by a loxP-stop-loxP drug selection cassette. Upon Cre mediated removal of the loxP-stop-loxP cassette, translation of the resulting transcript produces optimized Flp fused to estrogen receptor (Flpo-ERT2) (Lao, Raju, Bai, & Joyner, 2012). The transcript will also translate EGFP from an internal ribosomal entry site. While Flpo-ERT2 is expressed, the protein is excluded from the nucleus by the estrogen receptor fusion until tamoxifen administration.

To implement this system, loxP sites are engineered into a gene or genes of interest that will be modified first while gene(s) to be modified second are modified by *frt* sites (Figure 1b). The transgene targeted Gt(ROSA)26Sor allele (Gt[ROSA]26Sor^{tm1dwg}) is introduced via breeding into mice containing the loxP and *frt* modified genes and a source of Cre

expression is provided to initiate recombination, perhaps in the form of a Cre expressing virus or a transgene restricting Cre expression to a desired cell type. Cre expression creates the first gene mutation(s) while simultaneously removing the loxP-stop-loxP cassette allowing both Flpo-ERT2 and EGFP expression, thereby genetically marking Cre modified cells. Upon administration of tamoxifen at a time defined by the investigator, Flpo-ERT2 localizes to the nucleus where it has access to the genome and can mediate DNA recombination between frt sites, thus creating the second set of gene mutations. Flpo-ERT2 activity also deletes the EGFP transgene to distinguish cells containing the secondary mutation(s) by loss of EGFP protein.

The targeting vector was transfected into mouse embryonic stem cells (ES), and 66 neomycin resistant ES cell clones were screened by Southern blotting. BamHI restricted genomic DNA hybridized with a 5' flanking probe overlapping the Gt(ROSA)26Sor promoter (Figure 2a) is expected to generate a 4.8 kilobase pair (kbp) fragment for the correctly targeted allele while the original Gt(ROSA)26Sor allele generates a 5.8 kbp fragment. Twenty of 66 ES cell clones analyzed exhibited bands of both expected sizes (Figure 2b), indicating these 20 contained one correctly targeted Gt(ROSA)26Sor^{tm1dwg} allele. Gt(ROSA)26Sor^{tm1dwg} containing ES cell clones were injected into mouse blastocysts to generate chimeric mice, and chimeras mated with C57BL/6J mice to establish founders on a mixed 129/SvJae X C57BL/6J background. Germline transmission of Gt(ROSA)26Sor^{tm1dwg} was confirmed by Southern blotting (Figure 2c). Genotypes predicted from Southern blotting were validated by polymerase chain reaction (PCR) assays developed to specifically amplify the Gt(ROSA)26Sor or Gt(ROSA)26Sor^{tm1dwg} alleles (Figure 2a,d), and these assays were used for routine mouse genotyping using tail clip DNA. Mice heterozygous or homozygous for Gt(ROSA)26Sor^{tm1dwg} were generated at the expected Mendelian frequency, and mice appeared normal as no phenotype distinguishable from wild type was detected by gross observation over the lifetime of these animals. Thus the Gt(ROSA)26Sor^{tm1dwg} allele did not appear to impair normal murine development or homeostasis. To test for successful Cre mediated recombination, the Gt(ROSA)26Sor^{tm1dwg} allele was bred into mice containing a Meox2-Cre transgene. Meox2-Cre is expressed in the epiblast such that most cells of the developing embryo have been exposed to Cre activity (Tallquist & Soriano, 2000). Southern blot analysis of tail genomic DNA from such mice exhibit the predicted 6.1 kbp band predicted for the Cre recombined Gt(ROSA)26Sor^{tm1dwg} allele (Figure 2c).

We isolated mouse embryonic fibroblasts (MEF) from homozygous Gt(ROSA)26Sor^{tm1dwg} mice in order to validate proper function. MEF were infected with Cre expressing recombinant adenovirus (AdCre), treated with tamoxifen, or both. EGFP expression was assessed in live cells by fluorescence microscopy. EGFP was detected in AdCre treated MEFs, but not in untreated, tamoxifen treated, or AdCre+tamoxifen treated MEFs (Figure 3a). With efficient Cre delivery provided by adenoviral infection, EGFP is detected in nearly all cells. This indicates Gt(ROSA)26Sor^{tm1dwg} is working as designed and that expression of the bicistronic transcript is robust. To confirm the expected recombination events, genomic DNA was extracted from untreated or treated MEFs and analyzed by PCR using primers specific for the Cre recombined or Flpo-ERT2 recombined Gt(ROSA) 26Sor^{tm1dwg} allele (Figure 3b). As expected, infection with AdCre generated the expected DNA fragment

indicative of Cre mediated removal of the loxP-stop-loxP cassette (Figure 3c). The expected PCR band diagnostic of Flpo-ERT2 recombined Gt(ROSA)26Sor^{tm1dwg} only occurred in cells treated with both AdCre and tamoxifen; the larger 1.5 kbp band is the predicted size if Gt(ROSA)26Sor^{tm1dwg} has not undergone Flpo-ERT2 mediated recombination. By assessing the ratio of these two bands, it is apparent that the loxP-stop-loxP cassette blocks expression of Flpo-ERT2 effectively (tamoxifen only), and that Flpo-ERT2 mediated recombination can be quite efficient (AdCre plus tamoxifen). The 3' recombination junction was also verified using a reverse primer homologous to Gt(ROSA)26Sor, but outside the homology arms used in the targeting vector. PCR amplified a band of 795 base pair size from MEFs treated with AdCre+tamoxifen, a band size consistent with the Flpo-ERT2 recombined Gt(ROSA)26Sor^{tm1dwg} (Figure 3d). Although not efficiently amplified, a larger band consistent in size with that of the Flpo-ERT2 unrecombined Gt(ROSA)26Sor^{tm1dwg} could also be detected in the expected samples.

To validate function of Gt(ROSA)26Sor^{tm1dwg} in vivo, we generated PBCre4:Gt(ROSA)26Sor^{tm1dwg} mice. The PBCre4 transgene is expressed specifically in prostate epithelium in a mosaic pattern (Wu et al., 2001). Prostate tissue was dissected from these mice, either untreated or treated with tamoxifen, processed for frozen sections, and sections imaged by fluorescent microscopy to detect EGFP expression. Liver tissue from the same mice, or prostate tissue from wild type mice, were processed similarly to serve as negative controls. Prostate tissue in PBCre4:Gt(ROSA)26Sor^{tm1dwg} mice exhibited a mosaic of EGFP positive cells (Figure 4a), consistent with PBCre4 expression. We cannot formally rule out that mosaic EGFP expression is due to Gt(ROSA)26Sor^{tm1dwg}, but this is unlikely given the uniform EGFP positivity observed in vitro (Figure 3). Only background auto-fluorescence was detected in PBCre4:Gt(ROSA)26Sor^{tm1dwg} liver tissue or wild type prostate tissue. In tamoxifen treated mice, EGFP positive cells in the prostate were reduced or eliminated, with the number of EGFP positive prostate cells remaining varying from mouse to mouse. The efficiency of Flpo-ERT2 mediated recombination is likely influenced by the exposure of tissue to tamoxifen as in vitro experiments (Figure 3) demonstrate Flpo-ERT2 expression from Gt(ROSA)26Sor^{tm1dwg} does not limit recombination efficiency. PCR genotyping of tissue was also performed to confirm Gt(ROSA)26Sor^{tm1dwg} function (Figure 4b). As expected, the Cre recombined band was detected in prostate tissue from all PBCre4 mice, but the Flpo-ERT2 recombined band was only detected in mice treated with tamoxifen, even when using a sensitive nested PCR assay (Figure 4c). This indicates tamoxifen induced Flpo-ERT2 activation deleted the EGFP expression cassette as designed with no detectable Flpo-ERT2 activity in the absence of tamoxifen.

We describe the construction and functional validation of the Gt(ROSA)26Sor^{tm1dwg} allele targeted to the Gt(ROSA)26Sor locus. Gt(ROSA)26Sor^{tm1dwg} can be used with loxP and frt modified loci to control the order and timing of different genetic alterations in mice. Importantly, Flpo mediated recombination at frt modified loci only occurs in cells where Gt(ROSA)26Sor^{tm1dwg} has previously undergone Cre mediated recombination. This facilitates analysis of cell autonomous effects of the secondary mutation(s). The timing and extent of the secondary mutations is controllable by the schedule and dose of tamoxifen administration. This will be useful for testing time sensitive hypotheses, such as effects of secondary mutations during development or disease progression. By limiting the dose of

tamoxifen administered, genetic mosaics composed of mutated cells with and without the secondary mutation can be intentionally created to evaluate clonal dynamics and relative fitness. An analogous mouse allele for controlling expression of CreERT2 with an *frt*-stop-*frt* cassette has been described previously (Schonhuber et al., 2014), but this system is limited by the smaller number of *Flp* expressing transgenic alleles currently available. Gt(ROSA)26Sor^{tm1dwg}, on the other hand, leverages the large number of well characterized transgenes capable of restricting Cre expression to a particular cell type or tissue. While fewer *frt* modified gene alleles are available, relative to *loxP* modified genes, the advent of CRISPR/Cas9 genome editing technology has made construction of *frt* modified alleles considerably less time consuming. Thus Gt(ROSA)26Sor^{tm1dwg} will be useful in a wide variety of applications where independent control of multiple mutations is desirable.

3 | METHODS

3.1 | Construction of the Gt(ROSA)26Sor^{tm1dwg} allele

The Flpo-ERT2 cDNA (Lao et al., 2012) was subcloned into the *AscI* site of CTV (Xiao et al., 2007) to generate the targeting vector. CTV (Addgene #15912) is a modified form of the Rosa26–1 targeting vector with the CAG promoter driving expression of a *loxP*-neo/stop-*loxP* neomycin selection cassette and a *frt*-flanked IRES-eGFP cassette. Successful targeting vector construction was confirmed by DNA sequencing. The targeting construct was electroporated into 129/ SvJae ES cells and G418 resistant colonies screened by Southern blotting. Correctly targeted ES cells were used to construct germ line transmitting chimeras by blastocyst injection.

3.2 | Mouse genotyping and treatment

Transmission of the Gt(ROSA)26Sor^{tm1dwg} allele was confirmed by Southern blotting and PCR genotyping of genomic DNA extracted from tail biopsies. DNA was extracted from tail specimens clipped from recently weaned mice or from embryonic yolk sac tissue as described by Laird et al. (1991). Briefly, tissues were digested in Laird lysis buffer with protease K overnight at 55 °C. Undigested tissue was collected by centrifugation, and the supernatant containing genomic DNA precipitated with isopropanol. Southern blotting was performed using standard methods as described previously (Sun et al., 2006). Briefly, BamHI restricted genomic DNA was resolved by gel electrophoresis and blots hybridized to an 800 bp 5' flanking probe overlapping the Gt(ROSA)26Sor promoter. The expected structure of the 3' recombination junction was verified by PCR using primers MGRT3c-F and RosaWT-R. Routine genotyping was performed by PCR analysis of the 5' recombination junction using RosaCTV-F and RosaCTV-R (Gt [ROSA]26Sor^{tm1dwg}) or RosaWT-R (Gt[ROSA]26Sor). All PCR reactions were performed with Taq polymerase (Fermentas Inc., Hanover, Maryland) or Q5 high fidelity DNA polymerase (New England Biolabs, Ipswich, Massachusetts) in supplier provided buffer. PCR was initiated with a 2'30" denaturation cycle at 94 °C, followed by 12–30 cycles of 30 s denaturation, 30 s annealing at 61 °C, and 30 s extension at 72 °C. After the final 10 min extension at 72C, PCR products were resolved by agarose gel electrophoresis and stained with ethidium bromide. Oligonucleotide primers used for genomic DNA genotyping and the expected DNA fragment sizes are listed in Table 1.

3.3 | Mouse tamoxifen treatment and tissue processing

To initiate Cre recombination, the Meox2^{tm1(cre)Sor} allele (Tallquist & Soriano, 2000) or the PB-Cre4 transgene (Wu et al., 2001) were bred into Gt(ROSA)26Sor^{tm1dwg} containing mice. Mice were treated with tamoxifen essentially as described (Pitzonka et al., 2013); tamoxifen (Sigma-Aldrich, St. Louis, Missouri) at 20 mg/mL was prepared and 0.1 mL administered (70–75 mg/kg body weight) to 9–12-week-old mice once per day for 5 days via intraperitoneal injection. Weight was monitored during and after treatment. Mice were euthanized 12–24 days after administration of tamoxifen. All tissues were dissected immediately following euthanasia and processed for frozen sectioning as described (Sun et al., 2011). Briefly, tissue specimens were embedded in optimal cutting temperature (OCT) compound and cryosections cut at 5-μm depth. Tissue processing and sectioning takes 5–7 days before slides are imaged. Slides were briefly counterstained with DAPI prior to mounting and imaging. Gt(ROSA) 26Sor^{tm1dwg} containing mice are available for distribution to the research community upon acceptance of this manuscript for publication. All animal experiments were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee at the department of Laboratory Animal Resources, Roswell Park Comprehensive Cancer Center, Buffalo, New York.

3.4 | MEF isolation, culture, and treatment

Embryonic day 13.5 embryos were isolated from timed pregnancies. Once dissected from the uterus and analyzed to confirm gestational age, the head and liver are removed. Tissue is minced in PBS with trypsin and incubated for 10 min at 37 °C with gentle shaking. The disaggregated tissue is then passed through an 18.5 gauge needle to generate single cell suspensions. Cells are cultured in DMEM supplemented with 10% fetal bovine serum, 2 mmol/L L-glutamine, MEM nonessential amino acids, 2-mercaptoethanol, and Gentamicin at 37 °C in a humidified atmosphere of 5% CO₂ in air. MEFs are infected with Ad5CMVCre (University of Iowa Viral Vector Core) and/or treated with tamoxifen at 2 μM. Cells are imaged 5 days after treatment. For PCR analysis, DNA is extracted 2 days after treatment.

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FIGURE 1.

Design of the *Gt(ROSA)26Sor^{tm1dwg}* allele. (a) A schematic representation of the *Gt(ROSA)26Sor^{tm1dwg}* allele with the transgene inserted between exons 1 and 2 (numbered boxes) of *Gt(ROSA)26Sor*. Effects of Cre and Flpo-ERT2 mediated recombination on *Gt(ROSA)26Sor^{tm1dwg}* are shown. Thick lines represent genomic DNA, thin lines represent targeting plasmid DNA. CAG represents the promoter used to drive transgene expression, stop is the neomycin selection stop cassette containing transcriptional/translational termination signals, *FlpoER* is the cDNA expressing Flpo-ERT2, and *EGFP* represents an internal ribosomal entry site and the cDNA encoding enhanced green fluorescent protein. Triangles depict loxP recombination sites while ovals represent *frt* sites. (b) The strategy for controlling the order and timing of mutations in two genes is depicted in the drawing using symbols as in (a). Active transcription is indicated by perpendicular arrows

FIGURE 2.

Construction of Gt(ROSA)26Sor^{tm1dwg}. (a) A schematic representation of Gt(ROSA)26Sor, the Gt(ROSA)26Sor^{tm1dwg} targeting vector, the correctly targeted Gt(ROSA)26Sor^{tm1dwg} allele, and Gt(ROSA)26Sor^{tm1dwg} after Cre mediated recombination is shown with symbols as in Figure 1. The positions of the Southern blotting probe and diagnostic BamHI sites are shown with lengths between sites in kilobase pairs. Green and red arrows indicate the positions of polymerase chain reaction (PCR) primer pairs used for PCR genotyping of Gt(ROSA)26Sor and Gt(ROSA)26Sor^{tm1dwg}, respectively. (b) BamHI restricted genomic

DNA extracted from representative ES cell clones transfected with the targeting vector were analyzed by Southern blotting to screen for correctly targeted alleles. The position of molecular weight markers is at left in kilobase pairs with wild type (WT) and correctly targeted Gt(ROSA) 26Sor^{tm1dwg} (C) alleles indicated at right. Probe position and expected band sizes are indicated in (a). All five ES cell clones shown contain a band of the expected size for the correctly targeted Gt(ROSA) 26Sor^{tm1dwg} allele. (c) Chimeric mice were generated by blastocyst implantation of correctly targeted ES cells. Tail clip genomic DNA was extracted from descendants of these chimeric mice and analyzed by Southern blotting as above. Mice containing the MeoxCre transgene are indicated. The Cre recombined Gt(ROSA)26Sor^{tm1dwg} allele with predicted size of 6.1 kbp pairs is marked (R). (d) Polymerase chain reaction (PCR) assays designed to specifically amplify the Gt(ROSA) 26Sor or Gt(ROSA)26Sor^{tm1dwg} alleles using primers indicated in (a) were used to analyze tail clip genomic DNA. PCR amplified DNA was resolved by agarose gel electrophoresis and stained with ethidium bromide. A representative gel image is shown with pixels inverted for clarity. Molecular weight markers and band identity are as above. Expected band sizes are listed in Table 1

FIGURE 3.

Analysis of Gt(ROSA)26Sor^{tm1dwg} in MEFs. (a) MEFs were isolated from mice homozygous for Gt(ROSA)26Sor^{tm1dwg}, cultured in vitro, and treated with adenovirus designed to express Cre and/or tamoxifen to induce Flpo-ERT2 activity as indicated. Live cells were imaged by fluorescent microscopy to detect EGFP expression, and representative images are shown. Scale bars represent 50 μ m. (b) Drawing showing the position of polymerase chain reaction (PCR) primer pairs designed to detect Cre (green arrows) or Flpo-ERT2 (red arrows) mediated Gt(ROSA)26Sor^{tm1dwg} recombination. A 3' flanking primer

(blue arrow) was used to verify the 3' recombination junction of the targeted allele. Symbols are as in Figure 1. (c) Genomic DNA was extracted from Gt(ROSA)26Sor^{tm1dwg} containing MEFs treated as above and analyzed by PCR assays designed to specifically amplify Cre or Flpo-ERT2 recombined Gt(ROSA)26Sor^{tm1dwg} alleles. (d) DNA was also analyzed by a PCR assay using the 3' flanking primer. PCR amplified DNA was resolved by agarose gel electrophoresis, stained with ethidium bromide, and imaged. Predicted fragment sizes are in Table 1. Representative gel images are shown with pixels inverted for clarity. The position of molecular weight markers in kilobase pairs are shown at left

FIGURE 4.

Analysis of the $Gt(ROSA)26Sor^{tm1dwg}$ in vivo. (a) PBCre4: $Gt(ROSA)26Sor^{tm1dwg}$ (dwg) or wild type (wt) mice, either untreated or treated with tamoxifen (tam), were euthanized and the indicated tissues dissected and processed for frozen sectioning. DAPI counterstained frozen sections were imaged under fluorescent microscopy to detect EGFP. Representative images from at least five mice are shown. Scale bar represents 50 μm . (b) A schematic showing the position of polymerase chain reaction (PCR) primer pairs designed to detect Cre (green arrows) and Flpo-ERT2 (red arrows) mediated recombination of

Gt(ROSA)26Sor^{tm1dwg} is shown, with symbols as in Figure 1. (c) Prostate tissue genomic DNA was extracted from PBCre4:Gt(ROSA)26Sor^{tm1dwg} mice, either untreated or treated with tamoxifen as indicated. DNA was analyzed by PCR assays as in (b), including a nested PCR assay to detect the Flpo-ERT2 recombined allele (nFlpo). PCR amplified DNA was resolved by gel electrophoresis and stained with ethidium bromide. Predicted fragment sizes are in Table 1. A representative gel image is shown with pixels inverted for clarity. The position of molecular weight markers in kilobase pairs are shown at left

TABLE 1

Polymerase chain reaction primers used for DNA genotyping

Primer	Sequence	Purpose
RosaCTV-F	GGA GTA GGC GGG GAG AAG GC	Forward primer amplifies Gt(ROSA)26Sor ^{tm1dwg} (251 bp) and Gt(ROSA)26Sor (397 bp)
RosaCTV-R	CTC AAC CGC GAG CTG TGG AA	Reverse primer amplifies Gt(ROSA)26Sor ^{tm1dwg} (251 bp)
RosaWT-R	CTC CGA GGC GGA TAC AAG CA	Reverse primer amplifies wild type Gt(ROSA)26Sor (397 bp)
MGRT1-F	GTT CGG CTT CTG GCG TGT GA	Forward primer amplifies Cre recombined Gt(ROSA)26Sor ^{tm1dwg} (275 bp)
MGRT1-R	GCC TCT CGA ATC TCT CCA CG	Reverse primer amplifies Cre recombined Gt(ROSA)26Sor ^{tm1dwg} (275 bp)
MGRT3-F	TAC ATG CGC CCA CTA GCC GT	Forward primer amplifies Flpo recombined Gt(ROSA)26Sor ^{tm1dwg} (301 bp)
MGRT3-R	GCA ACT AGA AGG CAC AGT CGA G	Reverse primer amplifies Flpo recombined Gt(ROSA)26Sor ^{tm1dwg} (301 bp)
MGRT3c-F	CGT GGA GGA GAC GGA CCA AA	Forward nested primer amplifies Flpo recombined Gt(ROSA)26Sor ^{tm1dwg} (250 bp), together with Rosa ^{WT-R} amplifies across the 3' recombination junction (795 bp)
MGRT3c-R	AGG CTG ATC GGC CGC TCT AG	Reverse nested primer amplifies Flpo recombined Gt(ROSA)26Sor ^{tm1dwg} (250 bp)