



Short communication

Non-invasive Instant Genotyping of Fluorescently Labeled Transgenic Mice

Dieter Fink¹, Tien Yin Yau¹, Thomas Kolbe^{2,3} and Thomas Rüllicke¹

¹Institute of Laboratory Animal Science, University of Veterinary Medicine Vienna, Vienna, Austria; ²Biomodels Austria, University of Veterinary Medicine Vienna, Vienna, Austria; ³IFA-Tulln, University of Natural Resources and Life Sciences, Tulln, Austria

Summary

Fluorescence proteins have been useful as genetic reporters for a wide range of applications in biomedical research and are frequently used for the analysis of transgene activity.

Here, we show that expression levels of the ubiquitously expressed fluorescent proteins eGFP, mCherry and tdTomato can be measured in transgenic mouse lines with random or targeted integrations. We identified the tail of the mouse as the tissue best suited for quantifying fluorescence intensity and show that expression levels in the tail correlate with gene dose. This allows for instant non-invasive determination of the genetic condition at the transgenic locus (hemizygous/heterozygous and homozygous) while simultaneously providing an objective comparison for transgene expression levels among different mouse lines.

In summary, we demonstrate for the first time that the gene dose of a ubiquitously expressed fluorescence reporter can be reliably quantified and directly linked to the genotype of transgenic mice. Based on this information, animals with the appropriate genotype can be instantly selected without laborious analysis for establishing and breeding of new transgenic lines, reducing the number of “waste” animals. Furthermore, no tissue sampling is necessary, which is a significant refinement of genotyping procedures. Both aspects are important improvements for the genotyping of transgenic mice that follow the principles of the 3Rs (reduction and refinement).

Keywords: mCherry, eGFP, *in vivo* imaging, quantification, genotyping

1 Introduction

Cloning of the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* led to the discovery of a revolutionary new tool for molecular biology (Prasher et al., 1992). Fluorescent proteins have been used in biomedical research for labeling proteins, cell compartments, cells, tissues and whole animals (Chalfie et al., 1994; Okabe et al., 1997; Wiedenmann et al., 2009). Ubiquitously expressed, fluorescent proteins can be used for lineage tracing to determine transgenic offspring and to identify labeled cells or tissues for transplantation studies (Kretzschmar and Watt, 2012; Tyas et al., 2003). Numerous transgenic animals expressing a gene of interest linked to a fluorescent protein have been generated, simplifying the production and breeding of such fluorescently labeled animals. So far, only wild type and transgenic mice can be reliably distinguished using fluorescent proteins. We have previously shown that fluo-

rescence can be easily detected in the paws, ears, the nose and the tail of transgenic mice using an optical *in vivo* imaging system for small animals (Fink et al., 2010). Such systems also allow for the relative quantification of fluorescence emitted from transgenic mice.

Here, we tested whether the expression levels of fluorescent proteins in transgenic mice transcribed from a ubiquitous promoter can be reliably quantified and routinely used to determine the hemizygous/heterozygous or homozygous genotype of such animals. This gene dose effect has been noticed previously in newborn pups (Cao et al., 2005; Zhu et al., 2005) and adult mice (Cornett et al., 2011; Garrels et al., 2012). However, a reliable method for objective quantification of fluorescence protein expression has not yet been provided. To test our suggested technique for routine applications, we analysed three different homozygous viable transgenic mouse models produced by targeted or random transgene integration.

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Tab. 1: Animal numbers per group

Mouse line	Imaging position	Age (weeks)	Black coat colour			Albino			Figure
			+/+	+/m	m/m	+/+	+/m	m/m	
CAG-tdTomato	ventral/dorsal	7-9	7/7	6/6	4/4	5/3	5/3 ^(a)	5/3 ^(a)	Fig. 1b
			+/+	+/T	T/T	+/+	+/T	T/T	
UbC-eGFP	ventral	3-4	4	11	4	3	6	3	Fig. 1c
CAG-mCherry	ventral	5-7	6	5	4	3	4	4	Fig. 1d
CAG-mCherry	ventral	3-4	–	–	–	8 ^(b)	9 ^(b)	9 ^(b)	Fig. 1e

^(a) In the dorsal imaging group albino, each transgenic group (+/m and m/m) contained one female.

^(b) Group +/+ contained four, group +/m four, and group m/m five females.

In summary, we demonstrate for the first time that fluorescence reporter intensity in transgenic mice can be easily quantified with an *in vivo* imaging system. The results can be directly linked to the genotype and are suitable for the routine genotyping of fluorescently labeled transgenic mouse lines.

2 Animals, materials and methods

Animal care

Animal husbandry and care was in accordance with contemporary best practices and all individuals involved with the care and use of animals were educated and trained according to the Federation of European Laboratory Animal Science Associations (FELASA) category B requirements. Animals were housed in groups in open Makrolon cages in an optimal hygienic conditions (OHC) experimental unit under controlled environmental conditions (room temperature, $20 \pm 1^\circ\text{C}$; relative humidity, $55 \pm 10\%$; 12 hour light-dark cycle). They were supplied with a standard breeding diet (V1126, Ssniff Spezialdiaeten GmbH, Germany) and tap water *ad libitum*. Animals were free of pathogens according to FELASA recommendations and health status was monitored quarterly (Mähler et al., 2014). All animal experiments were discussed and approved by the institutional ethics committee in accordance with Good Scientific Practice (GSP) guidelines and national legislation.

Transgenic animals

B6;Cg-Tyr^{c-2J}/J-Tg(UBC-eGFP/ARR2PB-FLuc⁺)^{1VPC} mice (hereafter referred to as UbC-eGFP) were previously described (Fink et al., 2010). C57BL/6NcrJ-Tg(CAG-mCherry)^{690Biat} mice (hereafter referred to as CAG-mCherry) were produced by pronuclear injection of the SpeI-HindIII fragment containing the CAG-mCherry construct. Both mouse lines are available to the research community through the European Mouse Mutant Archive (EMMA). pCAG-mCherry was constructed by replacing the eGFP in pCAG-eGFP (obtained from Addgene, plasmid number 11150, Matsuda and Cepko, 2004) with a linker sequence adding NheI and MfeI sites and then cloning in the XbaI - EcoRI mCherry fragment excised from pFUCW (Fink et al., 2010). CAG-tdTomato

transgenic animals were obtained from The Jackson Laboratory [Gt(ROSA)26Sor^{tm4}(ACTB-tdTomato,-EGFP)^{Luo}/J, Stock No. 007576]. To obtain black-coated transgenic mice, animals were bred with C57BL/6NcrJ mice (Charles River, Germany). To obtain albino transgenic mice, animals were bred with albino C57BL/6J mice (obtained from The Jackson Laboratory, B6(Cg)-Tyr^{c-2J}/J, Stock No. 000058). For imaging, we selected the tail, a furless region of the body, to avoid any effects of the hair cycle on the thickness of the skin (Stenn and Paus, 2001). In addition, we compared images of the ventral and dorsal side of the tail. Animal numbers per group, age and sexes (male mice were used unless stated otherwise) are shown in Table 1.

Breeding scheme

All transgenic lines are maintained as homozygous mutants. To obtain the animals for testing our genotyping approach, homozygous animals were crossed with wild type mice. The resulting F1 offspring were then intercrossed to produce the F2 generation consisting of wild type, hemizygous/heterozygous and homozygous genotypes.

Identification, tissue sampling and euthanasia

For short-term identification, mice were marked with a permanent marker at the dorsal side of the tail immediately after removing the animals from the imager. For final analysis and confirmation of the genotype, mice were euthanized by cervical dislocation and tissue samples (tail) were taken from all animals for standard PCR genotyping and integration site determination.

Integration site determination

Integration site of the transgene in CAG-mCherry transgenic mice was identified using linker mediated PCR (LM-PCR) (Bressan et al., 2011). Briefly, genomic DNA from CAG-mCherry transgenic mice was digested with *Csp6I* for 5' LM-PCR and with *FspBI* for 3' LM-PCR. PCRs were performed with the first round primers *CAG-5-Out-Rev* (5'-GGAAAGTC-CCTATTGGCGTTA-3') and *Linker-Primer* (for the 5' joint) and *CAG-3-Out-For* (5'-TCCTCCTCTCCTGACTACT-3') and *Linker-Primer* (for the 3' joint). The second round primers were *CAG-5-Inn-Rev* (5'-TTATGTAAACGCGGAAGTC-CA-3') and *Nested-Primer* (for the 5' joint) and *CAG-3-Inn-For*



(5'-CCAGTCATAGCTGTCCCTCTTC-3') and *Nested-Primer* (for the 3' joint). The PCR cycling conditions for both rounds of the PCR were as follows: Initial denaturation at 95°C for 2 min followed by 25 cycles of 95°C for 15 s, 55°C for 30 s, and 72°C for 1 min and a final extension at 72°C for 7 min. The PCR reaction was run on an agarose gel and products were excised, gel extracted and sequenced using the *CAG-5-Inn-Rev* primer for the 5' joint and *CAG-3-Inn-For* primer for the 3' joint. Sequences obtained were blasted using the UCSC genome browser (<http://genome.ucsc.edu>).

Test breeding and PCR genotyping

Genotypes of UbC-eGFP transgenic animals obtained by fluorescence based genotyping were confirmed through test breeding.

Fluorescence based genotyping of the CAG-mCherry line was confirmed by placing a genomic primer upstream (*CAGmC-5-For*, 5'-GTTGGCTTGTTCCCTGTGAT-3') and downstream (*CAGmC-3-Rev*, 5'-TCTGTTTACTGTGAACACTTTGA-3') of the transgenic locus to amplify the 354 bp wild type band. The *CAGmC-5-For* and the internal primer *CAG-5-Inn-Rev* were used to amplify the 192 bp transgenic fragment. The PCR cycling conditions for CAG-mCherry genotyping reactions were as follows: Initial denaturation at 95°C for 2 min followed by 35 cycles of 95°C for 15 s, 55°C for 30 s, and 72°C for 1 min and a final extension at 72°C for 7 min.

Genotyping for the CAG-tdTomato targeted mutation was performed as described in Zong et al., 2005.

In vivo imaging (of the tail)

Mice were anaesthetized with isoflurane (Isoba, Schering-Plough Animal Health Corporation, UK) using the Xenogen XGI-8 Gas Anaesthesia System (Perkin Elmer). Initial isoflurane concentration and oxygen flow in the induction chamber was 5% and 1.5 l/min, respectively. After the mice were anaesthetized, the anaesthetic flow was switched from the induction chamber to the imaging unit and the isoflurane concentration was reduced to 2% (oxygen flow 0.1 l/min in imaging unit). The mice were kept under 2% isoflurane during the imaging procedure. Mice were imaged with the Xenogen IVIS 50 *in vivo* imager (Perkin Elmer) using the GFP or DsRed (for red fluorescent proteins) filter sets and an external halogen light source (DCR III, EKE 150 W, Polytec). Total area of the predefined region of interest (ROI) was set to 2 cm² (rectangular, 1 x 2 cm) and was placed at the proximal end, 2-3 mm below the base of the animal's tail. For background correction, one ROI was placed at the right bottom of the field of view, to be subtracted (see Fig. 1a). General settings for the imaging procedures were as follows: Binning M (medium = 4) except for albino CAG-mCherry and albino CAG-tdTomato mice, where binning HR (high resolution = 2) was used to avoid saturation; field of view (FOV) 12 and exposure time 1 s.

Statistics

Fluorescently labeled transgenic animals are easily distinguished from wild type animals using proper excitation light and filters. The main focus of this work is on fluorescence ef-

iciency levels of hemizygous/heterozygous and homozygous animals which show distinct differences that can be quantified. Signals that are measured for the wild type animals are background fluorescence of the tails. Therefore, a Student's t-test for group wise comparison of hemizygous/heterozygous and homozygous groups was performed using Microsoft Excel 2003 software (***, $p < 0.001$).

Software limitation and fluorescence light source intensity settings

The software (Living Image 2.0) allows the creation of regions of interest, and for simplification we created a 1 x 2 cm rectangular ROI that is copy-pasted and positioned over the tail as shown in Figure 1a. As the tail grows, the fluorescence efficiency increases as expected in response to our settings. This could be resolved by creating smaller regions of interest and positioning exactly over the tail. Due to limitations of the software, it is difficult to position a small ROI on the tail. The thickness of the line of the ROI cannot be adjusted and the colour coding of the ROI is similar to the colour coding of the fluorescence efficiency; therefore, the circles cannot be seen. However, our approach still allows for the measurement of genotype, if mice are imaged at approximately the same age/body size, and simplifies the genotype analysis.

The generally lower fluorescence efficiency of CAG-mCherry transgenic mice in Figure 1e compared to Figure 1d is due to a reduction of fluorescence excitation light intensity. The IVIS 50 is powered by an external halogen light unit where the intensity can be set within the range of 70-100%. To increase the life span of the lamp, the intensity was lowered for the longitudinal imaging. However, this did not influence the interpretation of our results.

3 Results

We first tested our genotyping approach to determine whether gene dose levels of a ubiquitously expressed fluorescent protein are distinct and if so, could this information be used to discriminate the genotypes of heterozygous and homozygous CAG-tdTomato reporter mice. This model was initially chosen due to the exact knowledge of the integration site of the transgene generated by homologous recombination into the ROSA26 locus (Muzumdar et al., 2007). Genotyping primers to identify wild type, heterozygous and homozygous CAG-tdTomato transgenic mice were readily available to confirm the results obtained from the fluorescence readout. In order to test any potential impact of the coat coloring, we measured the fluorescence efficiency of the same reporter locus on black and albino B6 mice. Positioning of the albino CAG-tdTomato mice (ventral view) and ROIs are shown in Figure 1a. Figure 1b demonstrates that the fluorescence efficiency in homozygous black coated and albino CAG-tdTomato transgenic mice (m/m) is almost two-fold higher than in heterozygous mice (+/m) with the same coat colour, suggesting a strong gene dose effect. The lack of skin pigmentation in albino mice results in a more than two-fold increase

of signal strength in both transgenic genotypes as compared to black coated mice. Ventral imaging of black coated mice shows a doubling of the fluorescent signal compared to the readings from dorsal imaging, which is due to less pigmentation of the ventral side of the tail (data not shown).

To further investigate the power of this technique, we applied the new genotyping procedure to the UbC-eGFP transgenic mouse line. Figure 1c shows the distinct efficiency levels of wild type, hemizygous and homozygous UbC-eGFP transgenic mice with different coat colours. Similar to CAG-tdTomato transgenic mice, the level of fluorescence efficiency of eGFP in

black-coated mice is reduced to less than half than in comparable albino mice. All genotypes obtained by fluorescence-based genotyping of the UbC-eGFP transgenic line were confirmed by test breeding (data not shown).

Our third fluorescent model expresses the red fluorescent protein mCherry from the same strong and common CAG promoter that was also applied in CAG-tdTomato mice (Niwa et al., 1991). This novel mouse line clearly shows distinct levels of fluorescence efficiency in hemizygous and homozygous transgenic mice. Black-coated mice give a lower signal compared to albino mice (Fig. 1d) as already shown for UbC-eGFP and

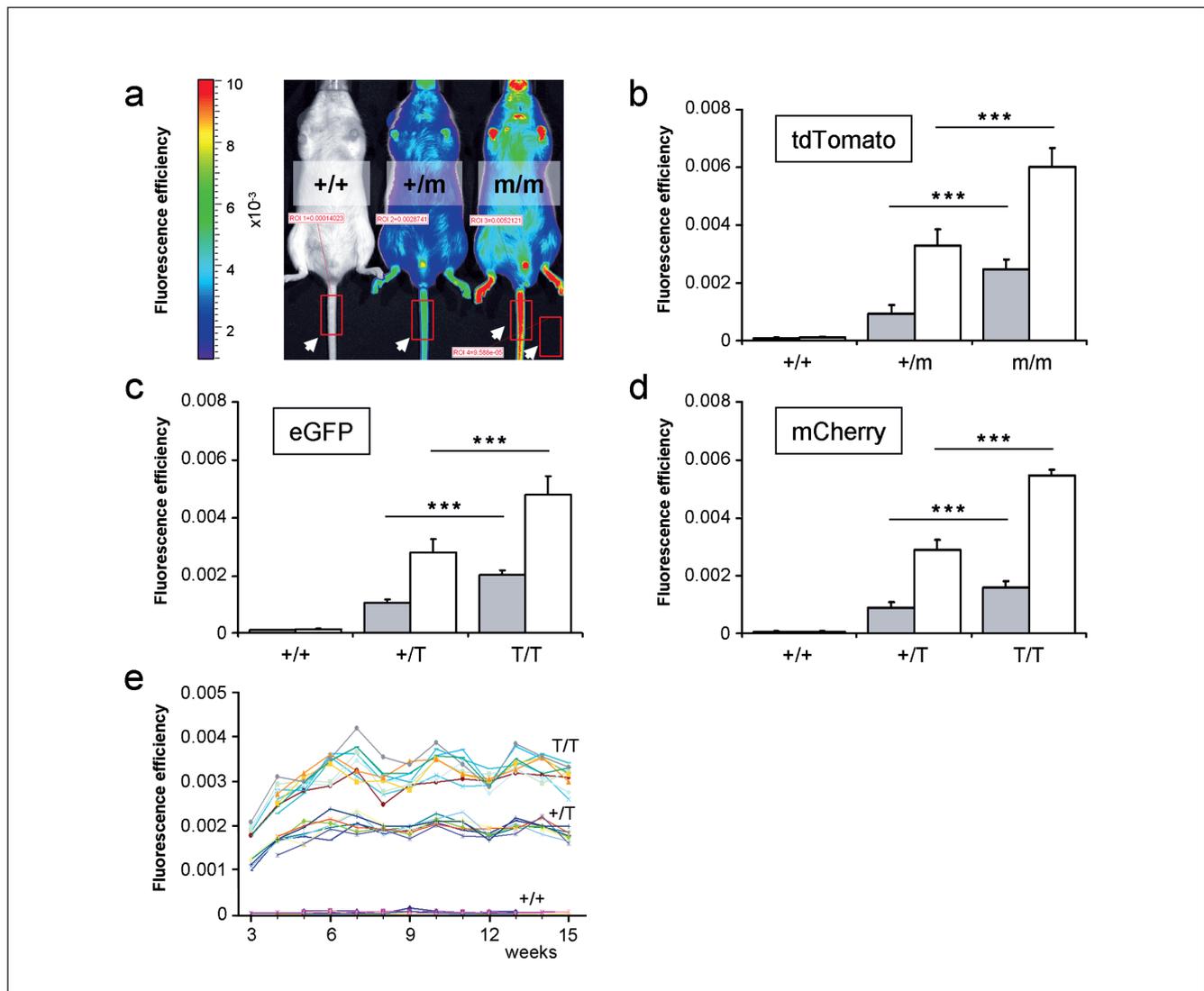


Fig. 1: Image and efficiency levels of fluorescently labeled transgenic mice

(a) Ventral view of albino CAG-tdTomato wild type (+/+), heterozygous (+/m) and homozygous (m/m) transgenic mice. ROIs are indicated by arrows. (b) Fluorescence efficiency levels of black coated (gray bars) and albino (white bars) CAG-tdTomato +/+, +/m and m/m transgenic animals. (c) Fluorescence efficiency levels of black coated (gray bars) and albino (white bars) UbC-eGFP wild type (+/+), hemizygous (+/T) and homozygous (T/T) transgenic mice. (d) Fluorescence efficiency levels of black coated (gray bars) and albino (white bars) CAG-mCherry wild type (+/+), hemizygous (+/T) and homozygous (T/T) transgenic mice. All graphs (b-d) show mean and standard deviation (***, $p < 0.001$). (e) Longitudinal imaging of albino CAG-mCherry transgenic mice. Relative fluorescence efficiency levels of hemizygous and homozygous transgenic mice are shown from 3 to 15 weeks of age. Each colored lined represents an individual mouse.



CAG-tdTomato mice. LM-PCR revealed that the transgene is located at an intergenic region on chromosome 13, locus 13qA2, not disrupting any known genes or transcripts. Primers were designed and all animals analysed by fluorescence based genotyping were confirmed by PCR genotyping (data not shown). Furthermore, albino CAG-mCherry transgenic mice can be easily distinguished by a trained person from wild type mice under daylight or artificial light (pink appearance of the skin of the tail, ears and paws) without the need for an emission and excitation filter (data not shown).

We further analysed the fluorescence efficiency levels of the CAG-mCherry mouse line over a period of time. Longitudinal imaging from 3 to 15 weeks reveals that the fluorescence efficiency levels between hemizygous and homozygous animals are maintained and do not change within this life span. The increase of fluorescence intensity from 3-6 weeks and the difference of fluorescence intensity in Figure 1d and 1e are due to our measurement settings which are explained in detail in the material and methods section.

4 Discussion

The aim of this study was to determine whether quantification of fluorescence intensity as a gene dose indicator is feasible for routine application to distinguish hemizygous/heterozygous from transgenic mice. Distinct fluorescence levels of different genotypes have been noticed before in newborn and adult transgenic mice ubiquitously expressing eGFP, Venus and mRFP1 (Cao et al., 2005; Garrels et al., 2012; Zhu et al., 2005). However, positioning and immobilization of newborn pups within an *in vivo* imaging device remains a challenge for accurate measurements. Alternatively to the ubiquitous expression, a tissue specific expression of fluorescent reporter in the eyes has been suggested for genotyping (Cornett et al., 2011). Compared to our standardised fluorescence based genotyping procedure, the above selection protocol requires specialised personnel to produce reliable and reproducible measurements. Furthermore, mouse lines with low transgene expression levels may not be suitable for simple visual examination as suggested by Cornett et al. (2011). Here, we provide the means for relative quantification of fluorescence intensity using different reporter mouse lines as a calibrator.

We have shown that this approach is suitable as an objective measurement for transgene expression levels in black coated and albino mice. Given that the black coated and albino mice represent the two fur and skin pigmentation extremes, we expect that this approach will be applicable to other coat color variations such as brown or agouti.

There are several transgenic mouse lines available which express reporter genes (<http://www.findmice.org>; Abe and Fujimori, 2013) and their number continues to increase. In this work, we tested three fluorescent transgenic mouse lines and show that this genotyping technique is feasible whether the fluorescent protein gene is randomly inserted or is integrated by homologous recombination into the common ROSA26 locus. It clearly shows that expression, driven by the CAG promoter

within the ROSA26, is gene dose dependent. So far, this approach has not failed on any mouse line tested with a single autosomal integration site. It may also be applicable for a transgene on the X chromosome due to a similar difference of the expression level after random X-inactivation (Hadjantonakis et al., 2001).

We are aware that this genotyping approach is currently suitable only for ubiquitous promoters due to the reliance on expression patterns of the tail. Fluorescently labeled blood cells circulating in the tail veins and arteries may also reveal the genotype of such mice if detectable with the imaging device. Additionally, fluorescent proteins expressed from tissue specific promoters that are visible in more transparent newborn pups may also function for determining the gene dose of such mice. However, this will require a new approach due to the current restrictions to *in vivo* imaging devices, which make them not suitable for newborns. The lowest exposure time setting on the IVIS 50 is 1 s for fluorescence imaging. Newer imaging devices have improved cameras and better suited filter sets that will definitely increase sensitivity and facilitate shorter exposure times. We are currently investigating the possibility of using a fluorescence stereo microscope to image moving newborn pups. If applicable, this would allow the determination of genotype immediately after birth without the need for immobilization or anaesthesia of the offspring. Deep tissue imaging utilizing improved red shifted fluorescent reporters like mCardinal may also be useful for transgenes driven by tissue specific promoters (Chu et al., 2014).

The presented genotyping technique may be applied to establish any transgenic line when the gene of interest is linked to a fluorescent reporter. This would allow an easy comparison of the transgene expression between parallel founder lines with the same transgene commonly produced by random transgenesis. Similar to the deletion of the antibiotic selection marker in gene targeting approaches, the reporter may be flanked by recombinase recognition sites and removed after the successful establishment of the transgenic line.

Concerns about transgene silencing and subsequent failure of this method may be raised. Considering the standard procedure for the establishment of transgenic lines produced by random integration, PCR genotyping identifies all founder lines including those that may not express the protein of choice. Quantification of fluorescence may represent a method for instant detection of transgene silencing. Therefore, with our method (similar to the use of western blotting), non-expressing lines can be excluded early from further breeding, resulting in a reduction of animal numbers.

In summary, we show for the first time that fluorescence efficiency levels in transgenic mouse lines ubiquitously expressing a fluorescent protein can be reliably quantified in the tail and linked to the gene dose of hemizygous/heterozygous or homozygous transgenic mice. The fluorescence efficiency levels are line specific and should be evaluated and confirmed for each transgenic mouse line before being used for genotyping. Importantly, this approach eliminates invasive tissue sampling (refinement of the experimental procedure). It also allows for early comparison of transgene expression between parallel founder

lines and of genotypes among offspring for fast establishment of homozygosity (reduction of animal numbers). Altogether, the application of fluorescent reporters is a useful scientific development to implement the principles of the 3Rs (Russell and Burch, 1959) in the genotyping of transgenic mice.

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Conflict of interest statement

The authors declare that they have no conflict of interests.

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Correspondence to

Dieter Fink, Dr nat. techn.
Institute of Laboratory Animal Science
University of Veterinary Medicine Vienna
Veterinärplatz 1
1210 Vienna, Austria
Phone: +43 125077 2805
e-mail: Dieter.Fink@vetmeduni.ac.at