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Genotyping gene-trap mutant mice by real-time PCR

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▼A major task in the second phase of the genome sequencing projects is the identification of coding sequence within the three billion base pairs of each the mouse and human genomes. At present, in addition to computer-aided programs, several high-throughput mutagenesis programs are being undertaken worldwide in order to achieve this goal (Ref. 1).

Gene-trap mutagenesis screens take advantage of random insertions into transcription units to drive a selection–reporter cassette and simultaneously to mutate the tagged genes. One of the advantages of gene-trap mutagenesis is that no *a priori* knowledge is needed about the structure of the tagged gene. However, because most gene-trap vectors contain splice-acceptor or splice-donor sites to capture translated gene sequence, the precise location of vector integration within introns is not known. Therefore, it is often difficult to generate external probes for Southern blots or external primers for PCR analysis in order to distinguish between homozygous and heterozygous mutants. To overcome this serious limitation in high-throughput mutagenesis screens, we developed a real-time PCR strategy that allows us to discriminate between mutants with either one or two copies of the gene-trap vector inside their genomes.

Real-time quantitative PCR is based on the quantification of a fluorescent dye [5′-6-carboxyfluorescein (5′-FAM)] that is quenched by 3′-6-carboxy-tetramethylrhodamine (3′ TAMRA) when attached to a probe located between two PCR primers but is activated by the 5′ exonuclease activity of the Taq DNA polymerase (Ref. 2, 3). Here, we describe a rapid method based on the quantification of a gene-trap vector relative to a standard locus within the mouse genome. This method allows the rapid genotyping of any gene-trap animal without prior knowledge of the mutated genes. Here, as an example, we describe the genotyping of a PT1βgeo insertion (Ref. 4) into the mouse *Neurochondrin* gene (Ref. 5) by comparing a multiplex PCR assay and a novel real-time-PCR-based method.

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1. Materials and methods

1.1. Taqman primers and probes

In order to quantify the inserted βgeo vector, primers and probes were designed that amplified a 83 bp fragment within the boundary of the *Escherichia coli* β-galactosidase gene (*lacZ*) and the stuffer sequence between *lacZ* and a neomycin resistance cassette (Fig. 1).

As a standard amplicon, we chose the murine Burkitt's lymphoma receptor gene 1 (*BLR1*; NM_007551). The human *BLR1* gene (NM_001716) is a two-exon gene that has been previously described as a single-copy standard for human genomic real-time PCR assays (http://www.appliedbiosystems.com/products/pdar.cfm).

Oligonucleotides and probes (Table 1) were designed using Primer Express (version 4.0; Applied Biosystems). Oligonucleotides and probes were synthesized by Interactiva (now Thermohybaid; http://www.thermohybaid.com/). Optimal oligonucleotide concentrations were determined using a primer matrix (not shown) to be 50 nM, and 250 nM for each TaqMan probe.

15 μl TaqMan Universal PCR Master Mix (Applied Biosystems) were mixed with 50 nM primer and 250 nM probe. Template DNA was prepared from mouse tails using the Qiagen genomic DNA kit according to manufacturer's instructions and dissolved in 200 μl TE buffer. In TaqMan assays, both 300 pg μl^{-1} and 1 ng μl^{-1} template DNA worked equally well.

The conditions for the TaqMan PCR reactions were 2 min at 50°C, 10 min at 94°C and 40 repetitions of 20 sec at 94°C, 20 sec at 55°C and 30 sec at 72°C.

The expected bands were 83 bp for PT1βgeo and 77 bp for BLR1. All expected fragments were obtained as single bands, as verified by agarose gel electrophoresis.

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Table 1. Real-time PCR primers and probes

Primer	Sequence	Location
PT1F	ctcctggagcccgtcagtatc	*lacZ*
PT1R	atcccctgacaccagaccaa	Stuffer–*lacZ*
PT1 probe	FAM–ATTCCAGCTGAGCGCCGGTCG–TAMRA	*lacZ*
BLRF	CGGAGCTCAACCGAGACCT	*BLR1*
BLRR	tgcaaaaggcaggatgaaga	*BLR1*
BLR probe	FAM–CTGTTCCACCTCGCAGTAGCCGAC–TAMRA	*BLR1*

Table 2. Multiplex PCR primers and probes

Primer	Sequence	Location
int6f	TCACTCCGACCTCTTACC	*Neurochondrin*
int7r944	AAGCAAGGGGTCAGGCTTAT	*Neurochondrin*
lac2	caaggcgattaagttgggtaacg	*lacZ*

Real-time PCR reactions were performed on an ABI Prism 7700 (Applied Biosystems).

1.2. Multiplex PCR primers

PCR conditions were as follows.

Hot Start Premix

4 μl 10× PCR buffer with NH_4SO_4
1 μl DMSO
1 μl of each 10 μM primer (Table 2)
33 μl H_2O
1 μl DNA template (1:100)

Taq–dNTP mix

1 μl 25 mM dNTP mix
1 μl 10× PCR buffer with NH_4SO_4
7.8 μl H_2O
0.2 μl 5 U ml^{-1} Taq (Fermentas)

PCR parameters

1. 95°C for 5 min
2. Hold at 82°C and add Taq–dNTP mix
3. 94°C for 30 sec, 55°C for 30 sec and 72°C for 2 min, repeated 30 times
4. 72°C for 7 min

Expected sizes were 1722 bp for the primers int6f and int7r944 (wild type), and 1566 bp for the primers int6f and lac2 (mutant).

2. Results

For the *Neurochondrin* mutation, we analysed 50 F2 generation animals using a multiplex PCR assay with one primer located inside *lacZ* and two external *Neurochondrin* primers (Fig. 2). As a preliminary result, both heterozygous and homozygous animals 3 weeks after birth were determined to lack any obvious defects (T. Floss *et al.*, unpublished).

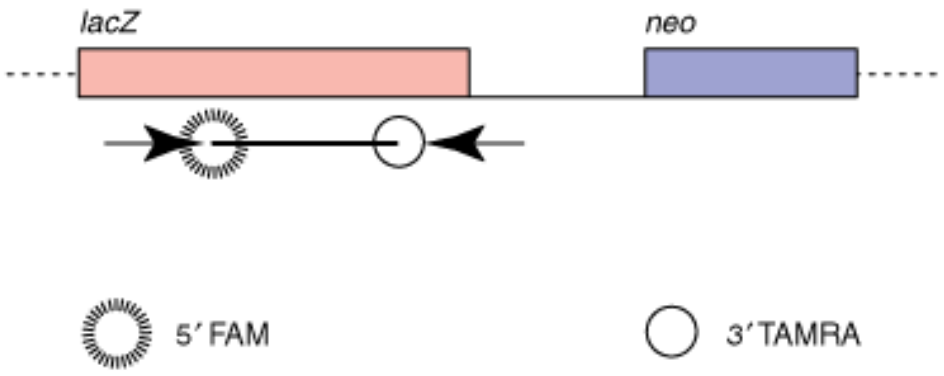

Fig. 1. The *lacZ* amplicon is 83 bp in size. Six base pairs of the 3′ PT1R primer overlap with the stuffer sequence between *lacZ* and the neomycin-resistance gene (*neo*).

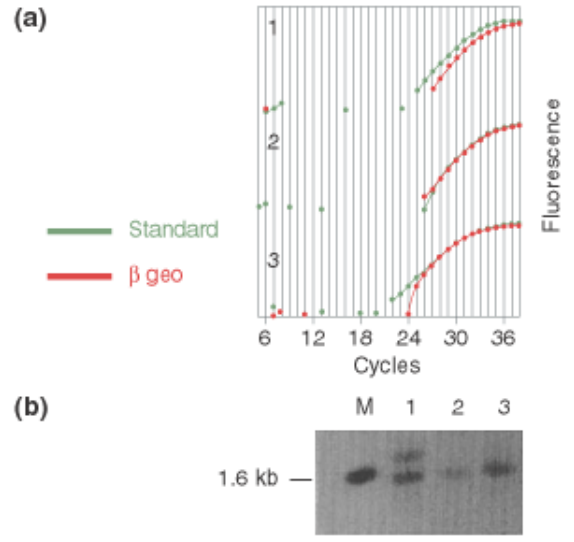

Fig. 2. Comparison of real-time PCR genotyping and multiplex PCR genotyping, using identical DNA templates from the gene-trap mouse strain W044B06 (*Neurochondrin*). (a) Real-time PCR. The *BLR1* standard is shown in green and the PT1 vector in red. (b) Multiplex PCR genotyping assay. Abbreviations: M, Marker; 1, heterozygote; 2 and 3, homozygotes.

Table 3. Threshold cycle (C_T) value differences between the BLR standard and the PT1 vector

Genotype	Average C_T difference	Expected C_T difference
+/–	1.1	1
–/–	0.08	0
–/–	No signal for PT1	n/a

When reanalysing 39 DNAs that had been previously genotyped by multiplex PCR using the real-time PCR assay (Table 3), we obtained average threshold cycle (C_T) value differences between the BLR standard and the PT1 vector of 1.1 for heterozygotes and 0.08 for homozygotes (the C_T value of a given reaction reflects the cycle in which the fluorescence is above the baseline with statistical significance). If two organisms have contents of the amplified fragment that differ by a factor of two, they would be expected to have C_T values that differ by 1. Therefore, the real-time PCR assay reliably distinguishes the different quantities within genomic DNA.

By Southern blotting using a *lacZ*-specific probe and DNA digests with two enzymes that either do not cut or cut once inside the vector, the *Neurochondrin* mutant was found to carry a single PT1 vector integration (not shown).

3. Discussion

We show that the relative quantification of genomic sequence by real-time PCR is a straightforward method for distinguishing between heterozygous and homozygous gene-trap animals (Ref. 6). C_T differences of 1 can be distinguished reproducibly and reliably, so we can determine whether a given animal has one or two copies of PT1βgeo in its genome.

The primers and probes described are suitable for the quantification of βgeo-type vectors, which are based on the pT1 gene-trap vector (Ref. 4). Multiple integrations were not genotyped reliably. Single-copy integrations are ideal for genotyping using this technique, and they can best be introduced using retroviruses. In order to determine whether a given mutant can be genotyped by real-time PCR, DNA from F1 animals should be included in the analysis. This method is now facilitating high-throughput mutagenesis using the gene trap or similar insertional mutagenesis methods.

This peer-reviewed article can be cited as: Floss, T., Uez, N., Frey, S. and Wurst, W. (2002) Genotyping gene-trap mutant mice by real-time PCR. *Technical Tips Online* t02593.

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Products Used

Taq polymerase: Taq polymerase from Roche Molecular Biochemicals

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