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# Real-time quantitative PCR-based system for determining transgene copy number in transgenic animals

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*In this paper, we describe a rapid and accurate real-time quantitative PCR-based system to determine transgene copy number in transgenic animals. We used the $2^{-\Delta\Delta Ct}$ method to analyze different transgenic lines without the requirement of a control sample previously determined by Southern blot analysis. To determine the transgene copy number in several mouse lines carrying a goat β-Lactoglobulin transgene, we developed a TaqMan® assay in which a goat genomic DNA sample was used as a calibrator. Moreover, we used the glucagon gene as a reference control because this gene is highly conserved between species and amplifies with the same efficiency and sensitivity in goat as in mouse. With this assay, we provide an alternative simple method to determine the transgene copy number, avoiding the traditional and tedious blotting techniques. The assay's discrimination ability from our results is of at least six copies and, similar to the limitations of the blotting techniques, the accuracy of the quantification diminishes when the transgene copy number is high.*

## INTRODUCTION

The pronuclear microinjection technique has been used as an important tool for the production of transgenic animals. However, this procedure is characterized by random integration of the transgene, usually as multiple copies in tandem that vary in number between transgenic lines (1). Traditionally, the transgene copy number in both founders and G1 hemizygous transgenic mice has been analyzed by blotting techniques (i.e., Southern blot analysis, dot blot, and slot blot), which are tedious and time-consuming methods that require a large amount of DNA sample for each assay. Moreover, the quantification by those methods is not accurate and gives ambiguous results.

With the emergence of the real-time quantitative PCR technology, different applications have been described for the analysis of transgenic organisms, such as the determination of transgene copy number in transformed plants (2) or the study of zygosity in transgenic animals (3,4).

Here we present a new application of the real-time quantitative PCR for transgene copy number determination in transgenic animals, characterized by the speed, high-throughput, sensitivity, and accuracy of the TaqMan® methodology (5,6). For this purpose, we used the $2^{-\Delta\Delta Ct}$ method (7) to compare the $\Delta C_t$ [cycle threshold ($C_t$); $C_t$ of target minus $C_t$ of control gene] value of transgenic animal samples with unknown transgene copy numbers with the ΔCt of a known calibrator. Unlike the approach used by Tesson et al. (3), the use of a control sample previously analyzed by Southern blot analysis is not required in the current method. A goat genomic DNA sample, carrying a single copy of the β-Lactoglobulin (*βLG*) gene, was used as a calibrator to determine the transgene copy number in several caprine *βLG* transgenic mice lines. Moreover, we describe the primers and TaqMan probe design that amplify with the same efficiency and sensitivity a highly conserved sequence of the glucagon gene in goat as in mouse samples that can be used for endogenous control gene amplification in both species.

## MATERIALS AND METHODS

### Animals and DNA Samples

Transgenic mice were previously generated by pronuclear microinjection of the pPX(7.0) construct, which con-

tained 410 bp of the goat *βLG* proximal promoter region, the entire transcription unit of the *βLG* gene, and 1.9 kb of the 3′ flanking region. Seven transgenic lines were obtained (Tg3, Tg19, Tg21, Tg29, Tg46, Tg60, and Tg56), and the number of transgene copies, ranging from 1 to 22, was estimated in these animals by Southern blot analysis (8).

For TaqMan copy number determination assays, we used genomic DNA isolated by the phenol-chloroform method (9) from the liver of founder and G1 hemizygous transgenic mice from the seven transgenic pPX(7.0) lines and from a goat liver. Prior to the assays, serial dilutions of genomic DNA samples (100, 50, 25, 12.5, and 6.25 ng) from a goat and a transgenic mouse (the founder of line Tg46, carrying one copy of the transgene as previously determined by Southern blot analysis) were prepared and used for validation of the method.

## Primers and Probes Design

PCR primers and probes were designed using Primer Express™ software (Applied Biosystems, Foster City, CA, USA) and are shown in Table 1. For the transgene amplification, the primers GOATPROF1 and GOATPROR1 were used to obtain an 88-bp-long product of the proximal promoter of the caprine *βLG* gene (GenBank® accession no. Z33881). The TaqMan MGB (minor groove binder; nonfluorescent quencher at the 3′ end) probe was GOATPRO-TQ. To normalize the amount of DNA added to the PCRs, we used a highly conserved single copy gene that could be amplified with the same efficiency and sensitivity in goat as in mouse. Nucleotide similarity searches in the GenBank database with Blastn showed that the glucagon gene is highly conserved between different species. However, because little information is available on the goat genome, we sequenced the exon 4 of the goat glucagon gene (GenBank accession no. AY588290) to confirm the conservation of the caprine sequence. We then designed the primers (GLCOE4F and GLCOE4R) that amplified a 130-bp-long product of the exon 4 of the caprine glucagon gene and the TaqMan MGB probe (GLCO-TQ).

## Real-Time PCR Procedure

All samples were run in triplicate in a 25-µL reaction volume containing 2× TaqMan Universal PCR Master Mix (Applied Biosystems), primers at a final concentration of 900 nM each, 250 nM probe, water to a 23-µL final vol-

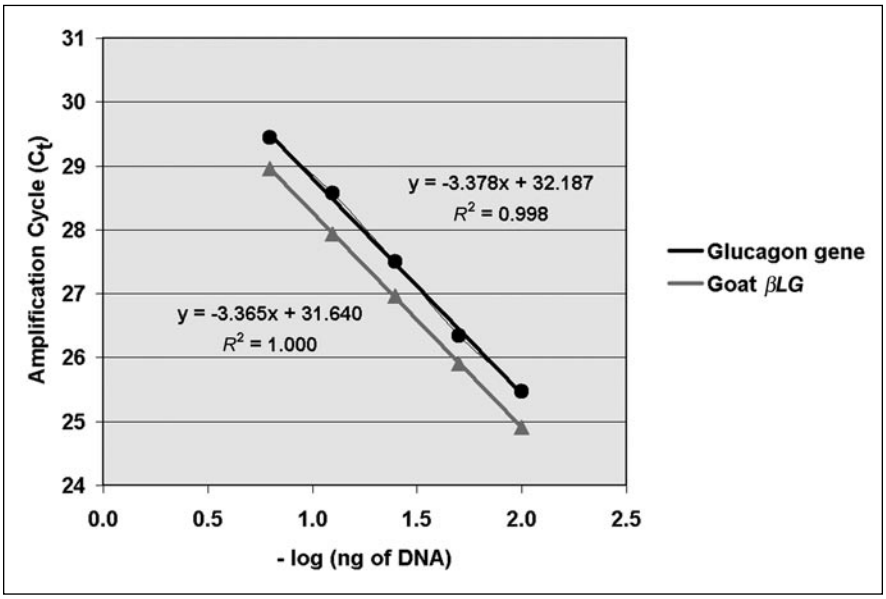


**Figure 1. Standard curve comparison.** Standard curves for the β-Lactoglobulin (*βLG*) transgene and the glucagon gene in serially diluted (2-fold) DNA samples from the transgenic founder animal of line Tg46, which carries a single copy of the *βLG* transgene. A very efficient amplification was obtained, as indicated by the slopes of the standards curves. $C_t$, cycle threshold.

**Table 1. Primers and Probes**

| Target Gene | Primers and Probes | Sequence |
|---|---|---|
| *βLG* | GOATPROF1 (Forward) | 5′-TGGAAGAAGGCCTCCTATTGTC-3′ |
| | GOATPROR1 (Reverse) | 5′-AGGTTCCCGGAATCCTACTTG-3′ |
| | GOATPRO-TQ (TaqMan MGB Probe) | 6FAM-5′-TCGTAGAGGAAGCCACC-3′ |
| Glucagon | GLCOE4F (Forward) | 5′-AACATTGCCAAACGTCATGATG-3′ |
| | GLCOE4R (Reverse) | 5′-GCCTTCCTCGGCCTTTCA-3′ |
| | GLCO-TQ (TaqMan MGB Probe) | VIC-5′-ACATGCTGAAGGGACC-3′ |

*βLG*, β-Lactoglobulin; MGB, minor groove binder; FAM, 6-carboxy-fluorescein.

**Table 2. Correlation Between TaqMan and Southern Blot Assays for Transgene Copy Number Determination**

| Transgenic Lines | Number of Copies by Southern Blot Analysis | | Number of Copies by TaqMan Assay | | |
|---|---|---|---|---|---|
| | G0 | G1 (n)[a] | G0 | G1 (n)[a] | SD[b] |
| Tg46 | 1 | 1 (1) | 1 | 1 (5) | 0.072 |
| Tg21 | 5 | 6 (1) | 3 | 3 (5) | 0.082 |
| Tg3 | 5 | 5 (1) | 4 | 4 (4) | 0.142 |
| Tg60 | 11 | 6 (1) | 7 | 6 (5) | 0.144 |
| Tg19 | 10 | 15 (1) | 16–18 | 18–20 (5) | 0.946 |
| Tg29 | 11 | N.D. | 14 | N.D. | N.D. |
| Tg56 | >20 | 22 (1) | ~40 | ~45 (4) | 5.217 |

~, approximately; N.D., not determined.
[a]Total number of transgenic mice analyzed in different lines by Southern blot analysis and TaqMan assay.
[b]Standard deviation of copy number determined among mice of the same transgenic line.

ume, and 2 µL of the genomic DNA. The PCR was run in the ABI PRISM® 7900 HT Sequence Detection System (Applied Biosystems) using the following amplification parameters: 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 s at 95ºC and 1 min at 60°C.

## RESULTS AND DISCUSSION

### Initial Validation of the Method

The use of the $2^{-\Delta\Delta Ct}$ method for relative quantification, a comparative technique in which a target gene is normalized to an endogenous control and relative to a calibrator, requires the PCR efficiencies of target and control genes to be approximately equal. Moreover, in our case, the use of a goat genomic DNA sample (with a single copy of the *βLG* gene) as a calibrator for transgenic mouse samples required that the PCR efficiencies and sensitivities of target and control genes in both species were similar. PCR efficiency can be calculated by plotting the $C_t$ as a function of $\log_{10}$ concentration of template (see Applied Biosystems user bulletin #2 at http://www.appliedbiosystems.com); the slope of the resulting trend line will be a function of the PCR efficiency, with a slope of -3.32 indicating that the PCR is 100% efficient.

To ensure that these requirements were met, prior to performing the TaqMan copy number assay, we generated standard curves for the transgene (*βLG* gene) and the endogenous control gene (glucagon gene) from both transgenic mouse and goat genomic DNA isolated from liver. We used this tissue because genomic DNA isolated from mouse tail biopsies using a standard proteinase K digestion method caused inhibition of real-time PCR (10). In mouse standard curves, a very efficient amplification was obtained, as indicated by the slope of the linear regression, and good correlation coefficients were observed (Figure 1). Slopes of -3.32 and -3.39 and correlation coefficients of 0.997 and 0.999 for the *βLG* and glucagon genes, respectively, were obtained in the goat (data not shown). Moreover, an absolute slope of 0.0638 (<0.1) was obtained in the goat when plotting the log input amount versus $\Delta C_t$, permitting the use of the $2^{-\Delta\Delta Ct}$ method for the relative copy number quantification (see Applied Biosystems user bulletin #2).

### TaqMan Copy Number Assay

TaqMan copy number determination assay in founder and G1 hemizygous pPX(7.0) transgenic mice was performed by the $2^{-\Delta\Delta Ct}$ method using the glucagon gene as an endogenous control and a goat sample as a calibrator. The number of transgene copies had been previously estimated in these animals by Southern blot analysis (8), which permitted the validation of this method. Moreover, to determine the accuracy of the TaqMan assay, four to five G1 mice were analyzed for each transgenic line, except for line Tg29 because the founder animal was a mosaic and did not transmit the transgene. In the transgenic samples, evaluation of $2^{-\Delta\Delta Ct}$ indicates the fold change in copy number of the goat *βLG* gene relative to the goat sample used as the calibrator. Because founders and G1 transgenic animals were hemizygous for the transgene while the goat sample (calibrator) was homozygous for the *βLG* gene, all results obtained were multiplied by a factor of two. The estimation of transgene copy number by the TaqMan assay was similar to the results previously obtained by Southern blot analysis in lines with a low copy number (<10) (Table 2). However, differences in the prediction of the number of copies were detected in transgenic mice from line Tg21 and in the founder of line Tg60 and can be attributed to the limitations of Southern blot quantification. Based on the equation that describes the exponential amplification of PCR,

$$X_n = X_0 \times (1 + E_x)^n \qquad \text{[Eq. 1]}$$

the highest $C_t$ difference between consecutive copy numbers is between one and two copies ($\Delta C_t = 1$). This $C_t$ difference diminishes as the copy number increases, thus reducing the accuracy of the quantification when the copy number is high. As expected, standard deviation (SD) of the copy number between mice of the same transgenic line is higher as the number of copies increases

(Table 2). In this study, we demonstrate that the assay's discrimination ability is of at least six copies (SD of 0.144 in line Tg60). However, difficulties in differentiating between sequential copy numbers are shown in line Tg19, which has 18–20 copies of the transgene and an SD of almost 1 (0.946). On the other hand, when the copy number is very high (line Tg56; approximately 40 copies), the $C_t$s for the transgene are out of the linear range of quantification, which makes it inaccurate.

In conclusion, we present a simple method for the determination of the transgene copy number, based on the methodology described by Tesson et al. (3). In this publication, the authors used a sample of known zygosity previously determined by Southern blot analysis to discriminate between heterozygous and homozygous transgenic animals. In contrast, in the present work, we can determine the transgene copy number in several mouse lines without the requirement of a known copy number mouse sample. We used as a calibrator a DNA sample from the species in which the transgene was generated and the glucagon gene as an endogenous control. Although this method has been developed in transgenic mice for a caprine *βLG* transgene, it can also be applied in transgenic animals from other species and carrying different transgenes. In fact, the glucagon's primers and probe design reported here can be used directly for the analysis of transgenic mice for transgenes with different ruminant sequences because an exact match of primers and probe exists in mouse and ruminant species. However, a single nucleotide mismatch exists both in human and pig glucagon genes for one of the primers used. The real-time quantitative PCR-based system described here represents a rapid, reliable, and accurate new tool for transgene copy number determination without the requirement of a control sample previously quantified by blotting techniques.

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## COMPETING INTERESTS STATEMENT

*The authors declare no competing interests.*

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