Accurate method for determination of transgene copy number using Real-time PCR in rabbits

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ABSTRACT

The transgene copy number can deeply influence the level of transgene expression and the ease of stabilizing expression in following generations.
It was so necessary to develop a method for determination of the inserted transgene copy number in transgenic rabbits. A real-time PCR assay was designed by Scanelis for detection and quantification of a target sequence used by Bioprotein Technologies in several genetically modified lines (WAP gene promoter).
To normalize the input amount of chromosomal DNA, Scanelis developed a second real-time PCR assay to detect and quantify an endogenous gene.
The $2^{-\Delta\Delta Ct}$ method, commonly used for gene quantification, assumes that both PCR efficiencies (WAP gene promoter and endogenous gene) are the same. Efficiencies were compared by plotting ΔCt against log dilution, and then considered very similar, allowing us to use the $2^{-\Delta\Delta Ct}$ method for transgene copy number calculation.
Wild type rabbits naturally contain two WAP gene promoter copies per cell and can so be used as calibrators.
Animals with a known transgene copy number (≠ 0) were not available. Thus, three experiments were performed in order to validate the method.
Firstly, we performed 12 replicates for each assay on samples from 5 wild type rabbits, as unknown samples. Each sample was considered as a calibrator too. 25 data sets were then obtained, for which the predicted transgene copy number was 0. This experiment was repeated three times. Average copy numbers and standard deviations were calculated for the 75 data sets. Conclusion was always 0 copy of transgenes.
Secondly, synthetic transgenic samples were constructed, with 1, 2, 6 or 14 transgene copies and tested with the developed method. Results were totally concordant with the predicted numbers.
Finally, a high reproducibility was observed on field samples and it was proven that the choice of calibrator has no effect on results.
All the results were statistically analyzed, depending on the replicate number taken into account (4 to 12) to evaluate the effect of this number on the accuracy of the applied method.
It would be now interesting to compare these results with other methods (Southern Blot analysis and determination of integration site number).
Thus, Real-time PCR enables very accurate quantification of the transgene copy number normalized to an endogenous reference and relative to a calibrator in transgenic rabbits.

INTRODUCTION

When generating transgenic animals, a first step in their characterization is to estimate how many copies of transgene have been integrated in the rabbit genome.
It was decided to develop a method based on real-time PCR and we hoped to reach high accuracy and reproducibility for estimates of the transgene copy number.

MATERIALS & METHOD

- **Samples**

Biopsies from wild type (wt) and transgenic rabbits collected by Bioprotein Technologies

- **Real-time PCR assays**

- Two real-time PCR assays for specific detection of the WAP Promoter (PROM) and an endogenous gene (REF) respectively
- PCR optimization for maximum reproducibility of replicates
- For each PCR assay the linearity was checked and the efficiency assessed (standard curves)
- The slope of log input amount vs. ΔCT was measured: it must be < 0.1 to allow the use of the ΔΔCt calculation for the relative quantification of target

- **Synthetic transgenic samples**

An absolute quantification of the WAP promoter was performed on a wild type sample. Different quantities of a plasmid containing the WAP promoter sequence were added to this wild type sample.

- **Method**

The developed method enables to estimate the WAP promoter (PROM) copy number of a transgenic sample, normalized according to a reference gene (REF) and relative to a calibrator (sample for which the PROM copy number is known).
The integrated transgene copy number per cell is then calculated (see Figure I).
The T< et T> values (95 %) are $(2^{-\Delta\Delta Ct+2\sigma} \times 2) - 2$ and $(2^{-\Delta\Delta Ct-2\sigma} \times 2) - 2$ respectively.

Figure I: Experimental determination of the transgene copy number according to the developed method

Nucleic Acids extraction from rabbit biopsies
"Macherey Nagel Viral RNA Isolation Kit" adapted protocol

Real-time PCR Inhibition Control

	Real-time PCR PROM		Real-time PCR REF	
	Sample	Calibrator = wt rabbit	Sample	Calibrator = wt rabbit
copies / cell	???	2	2	2

The transgene copy number T is then:

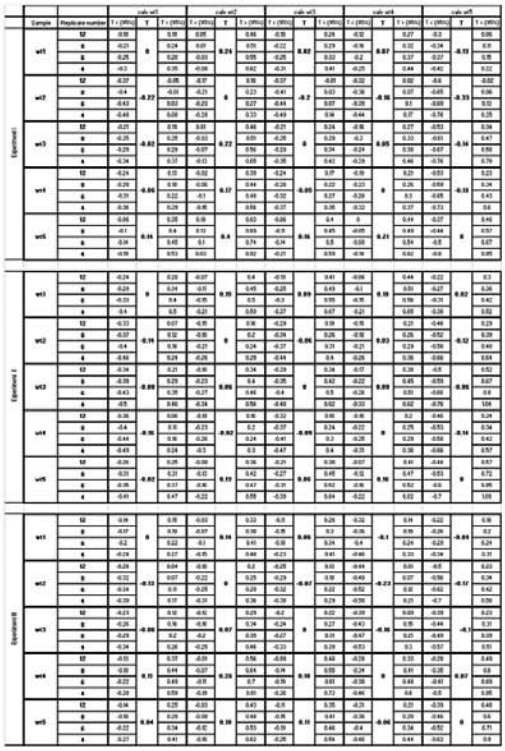
$$T = (2^{-\Delta\Delta Ct} \times 2) - 2$$

with ΔΔCt = (Ct PROM - Ct REF) sample - (Ct PROM - Ct REF) calibrator

RESULTS

The experimental value of the slope of log input amount vs. ΔCt is 0.0098 and so < 0.1. We were allowed to use the ΔΔCt method for our works.

Table 1: Real-time PCR estimates of transgene copy number for wild type rabbits (known samples)



> For 100 % of these 75 data sets, conclusion is 0 transgene

Table 2: Real-time PCR estimates of transgene copy number for synthetic transgenic samples

	Experiment I			Experiment II		
	T<	T	T>	T<	T	T>
wt+0	-0.13	**0.00**	0.14	-0.08	**0.00**	0.09
wt+1	0.73	**1.00**	1.30	0.85	**1.00**	1.16
wt+2	1.74	**2.07**	2.44	1.96	**2.17**	2.41
wt+6	5.25	**6.14**	7.16	6.07	**6.68**	7.34
wt+14	14.13	**15.74**	17.52	15.32	**16.50**	17.77

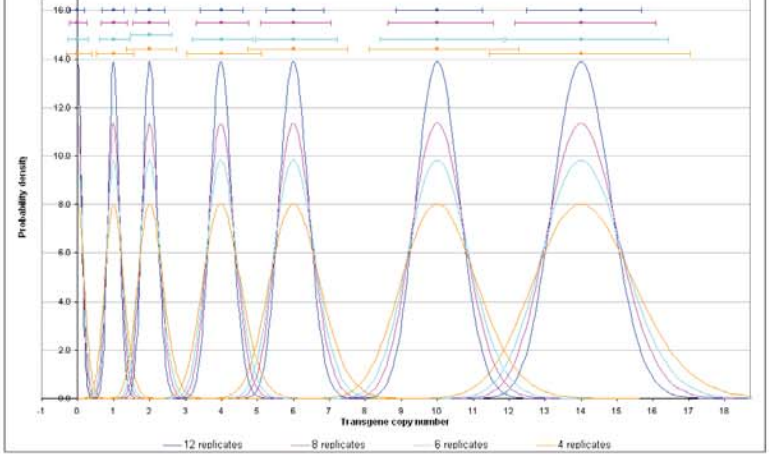
> High accuracy and reproducibility on synthetic transgenic samples

Table 3: Real-time PCR estimates of transgene copy number for transgenic rabbits

Sample	Experiment I			Experiment II			Experiment III			Experiment IV		
	T<	T	T>	T<	T	T>	T<	T	T>	T<	T	T>
1	9.09	**10.04**	11.08	9.74	**10.69**	11.71	10.76	**11.96**	13.27	9.82	**10.86**	12.00
2	7.22	**8.58**	10.14	8.09	**8.92**	9.81	7.55	**8.67**	9.93	8.32	**9.25**	10.25
3	7.04	**8.16**	9.41	7.62	**8.26**	8.95	7.11	**8.01**	9.00	7.11	**8.09**	9.16

> High accuracy and reproducibility on transgenic rabbit samples

Figure II: Probability density and the 95 % confidence interval depending on the number of replicates: average ΔΔCt standard deviation



> Measure accuracy is depending on the number of replicates for each PCR assay

CONCLUSION

Real-time PCR enables very accurate quantification of the transgene copy number normalized to an endogenous reference and relative to a calibrator in transgenic rabbits.