

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



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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

It was so necessary to develop a method for determination of the inserted transgerie copy infinite in transgeria. About A Fear-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-ΔΔC 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-ΔΔC 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 threat times. Average copy numbers destanded valuations were calculated for the 75 data sets. Conclusion was always 0 copy of transgenes.

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. Δ CT was measured: it must be < 0.1 to allow the use of the $\Delta\Delta$ Ct 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

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



	Real-time	PCR PROM	Real-time PCR REF					
	Sample	Calibrator = wt rabbit	Sample	Calibrator = wt rabbit				
cell	777	2	2	2				

The transgene copy number T is then: $T = (2^{-\Delta \Delta Ct} \times 2) - 2$ with ΔΔCt = (Ct PROM - Ct REF) - (Ct PROM - Ct REF) sample

RESULTS

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

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

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	Earspie	Pleplicate number	Tir (Minc)	T	T + (Mfr.c)	f + pring	T	t + princ	T 4 (MINO)	T	1 y fago 0	T / (MIN)	T	1 + prinq	Yeghtiq	T	TADMO
		U	410		1.0	101		0.86	4.9		0.24	49		1.27	4.1		6.06
	0.54.0		433		0.24	601	100	801	422	100	5.79	4.8	Section	635	-0.34		6.0
	with		425		121	931	8.24	0.55	425	8.82	9.22	0.2	8.87	9.37	427	-8.15	4.8
	20000		43		635	438		0.62	631		341	425	1000	1.44	440		8.22
	-	· v		-	48		-			-			-			_	
- 1			427			40		18	437		421	831		107	44	4.33	402
	wit		44	422	4.01	4.21		9.23	-0.41	42	1-03	438	416	9.07	-645		6.64
	1.54		-841		101	421	1 -	0.27	-644	7	9.07	428	7,77	8.5	-0.00	1	8.00
	g - 3		-648		608	-626		630	-0.49		0.36	-0.44		0.37	-6.76		6.25
	-	- 0	623		4.9	3.01		0.46	-621	$\overline{}$	9.24	48		6.27	-851	4,14	8.34
1.8	10000		435	550	6,28	421	1	101	4.26	100	9.29	42	100	0.33	-011		647
No.	*13		429	4.62	129	417	8,77	0.56	429		124	0.74	4.65	0.38	-847		150
8	24500	-	434		638				436			429		0.46			6.76
100				-		40	_	0.65			0.42		-		-0.7%	_	
- 1	100	tt	-824		0.00	4.80		6.39	424		3,17	4.9		821	-051		1.23
			628	4.05	0.10	0.00	8.57	0.66	428	4.05	0.22	0.23		0.26	-0.00	4.0	934
	911		-8.31	***	8.22	-61		0.48	432		827	-629		8.5	-645	1	643
			4.36		6.29	4.6	1	0.06	4:17	1	1.36	432		9.37	0.72	1	.04
- 1	_	u	4.04	-	636	1.0		9.63	4.06	-	0.4	0		0.44	407	-	0.40
	17.00		41		8.4	810		205	41		1.45	am	6	0.61	-044	1	857
	with			8.85			8.4						15.0				
	(23%)		438	100	148	4.1	1	0.74	-6.14		4.5	-0.50		0.54	4.6	1	447
\Box			0.99		853	8.00		SHE	427	_	233	4.11		141	48	_	325
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		ti.	421		6.29	437		0.4	49		9.41	498		0.44	427		83
			429		6.04	10.01	1	0.45	445	1.3	0.43	41	E	481	441	1	636
	well .	-	431		24	en.	9.75	45	41	9.89	1.15	48	9.00	150	431	8.62	5.67
	3					441	1	0.50									
	_		-0.4	_	4.8		_		4.27	_	847	421	-	0.88	438	_	1.52
		tr :	4.33		667	48		930	421		0.99	4.1		0.21	-046		829
	wil		4.37		1.12	48		65	4.34	4.00	936	-411	0.00	0.28	452	4,0	6.29
	444		-04	4.11	0.96	421	٠.	9.24	437	-	9.75	4.0	***	9,29	-0.04		0.40
		-	4.86		824	4.26	1	1.29	4244		0.4	425		1.76	-0.64		1.54
. 1	_	- 0	434	-	6.21	4.0		0.34	429	-	0.24	4.0		6.38	45		6.52
181			439		6.29	-0.23	1	84	425		142	A22		5.45	431		147
permet	WE2	-		4.00			8.89										
181	1 3		(84)		636 -627 -646 -64		- 0.5	426 681			-044		0.6				
171			45		346	-0.34		8.54	-540		0.62	-830		182	476		104
		10	0.36		806	4.0		16	. 632		3.00	4.6		8.2	446		934
	- 3	•	-24		11	A22	0.02	6.2	437	***	834	422		6.75	-8.53		8.34
	444		444	**	0.9	0.26		026	(0.41		9.2	9.25		0.29	416		0.62
		-	-441	1	824	43		63	447		0.4	421		0.76	-546		8.57
	_			_						-			_			-	882
		w	438		8.26	-616		0.38	421	1.86	8.36	-881		8.81	AH		
	wis		9.21	4.62	6.31	4.0	*"	0.42	427		0.46	4.0		5.47	-661		8.72
			435	***	833	4.6		0.17	431		552	4.6		982	44		135
			-0.41		6.47	4.22		0.55	-6.39		0.84	422		6.82	47	1	3.00
													$\overline{}$			_	
		- 10	0.19		0.8	4.81		9.33	4.1		128	4.22		EH	441		0.96
	1 8		Alt		4.9	A17		136	AM		63	4.8		100	428		62
	wtf		42		622	41	0.14	940	4.8	9.66	9.34	44	41	624	629	-0.00	824
			428		827	46	1	0.00	423		341	400		6.31	434		0.31
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		12	424		9.04	4.6	1	6.2	425		9.0	244		9.00	-85		4.23
	•tf		430	4.12	997	4.21		0.25	429	4.07	-0.00	-0.49	422	9.07	-016	-	634
	-		434	****	8.0	428	1	0.29	4532		9.22	+52		8.0	-042	1	642
			479		6.12	431	1	0.36	430	1	529	438		8.25	47	1	606
100	_	w	421	-	49	45		6.09	41	_	9.22	431	-	101	429	_	643
percent						44	1			1				15	-044	1	631
-81	w13		435	4.00	0.36		a.or	0.34	434		627	4.0	4.16			-41	
LÆ.			421	1	12	42	1	9.30	427		9.11	-0.67		140	-649		8.09
-			-634		626	-0.29		0.46	430		838	453		83	457		9.81
1		12	411		837	4.00		0.54	4.01		0.48	438		8.35	438		0.49
	3550		9.88	100	644	0.87	1	0.64	0.16		0.95	0.34	1	6.01	636		0.6
	***		AZZ	4.0 544 437	0.26	6.7	411		381	438		0.41	441	8.87	101		
	Section 2	- i	439		0.59	48	1	3.85	438		0.72	GM	1	84	45		135
	_			_			_						_			_	
	9	it.	434		928	4.81	1	6.43	4.8		h.16	431		821	439	1	EAB.
	wit		-0.90		829	438		0.48	-em	*"	9.41	836		0.20	446		9.6
		4	431	1	634	48	1 ""	0.53	-6.19	***	0.46	-01		6.34	450	٠.	6.71
	- 1		9.27	1 1	0.41	4.0	1	8.62	435	1	254	346		0.66	-842	1	0.0
				_			_		_	_	_		_	-	_	_	

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

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.

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

copies / c

		Experiment	1	Experiment II					
	Te	T	T>	T <	T	T>			
Wt+D	-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			

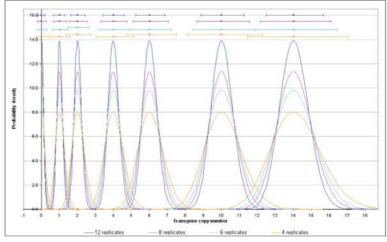
> High accuracy and reproducibility on synthetic transgenic samples

💌 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>	Ts	T	1>
1	9.09	10.04	11.08	9.74	10.69	11.71	10.75	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 $\Delta\Delta$ Ct standard deviation



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