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Title: Rapid zygosity determination in mice by SYBR Green real-time genomic PCR of a crude DNA solution

Authors; Takayuki Sakurai[1,*], Akiko Kamiyoshi[1], Satoshi Watanabe[2], Masahiro Sato[3], Takayuki Shindo[1]

Affiliations; [1]Department of Organ Regeneration, Graduate school of Medicine, Shinshu University, 3-1-1 Asahi, Matsumoto, Nagano 390-8621, Japan.
[2]Animal Genome Research Unit, Division of Animal Science, National Institute of Agrobiological Sciences, 2 Ikenodai, Tsukuba, Ibaraki, 305-0901, Japan.
[3]Section of Gene Expression Regulation, Frontier Science Research Center, Kagoshima University, 1-21-20 Korimoto, Kagoshima, Kagoshima 890-0065, Japan.

Full address for correspondence; *Takayuki Sakurai, Department of Organ Regeneration, Graduate school of Medicine, Shinshu University, 3-1-1 Asahi, Matsumoto 390-8621, Japan
Tel: +81-263-37-2578; Fax: +81-263-37-2564
Email: tsakurai@sch.md.shinshu-u.ac.jp

Abstract
We examined whether crude DNA extracts prepared from gene-engineered mouse tissues are suitable as a template for zygosity determination by SYBR Green real-time genomic PCR. A crude DNA solution was prepared by brief incubation with lysis buffer containing ear, tail, or fetus of ROSA26 mouse, a gene-trapped strain carrying the β-galactosidase (β-gal) gene. Five serially diluted crude DNA samples (original, 2-, 4-, 8-, 16-diluted) were next prepared and then subjected to three-step (95°C, 60°C and 72°C) reactions of real-time PCR to detect the β-gal gene and the receptor-activity-modifying protein 3 (ramp3) gene (as an internal reference gene). The slopes of standard curves obtained from the real-time PCR indicated that amplification efficiency was approximately 99%, and the efficiencies of target and reference were almost equal. With this system, we next determined the zygosity of mice derived from mating heterozygous ROSA26 females and males, and found a sharp distinction in zygosity, wild-type, heterozygous and homozygous. Assessment of crude DNA samples from other gene-engineered mice including B6ZP3Cre-Tg, B6rAM-Tg, and Ramp2-gene-targeted strains revealed that our method was effective for determination of zygosity. The present method is more convenient and rapid than formerly published methods employing purified genomic DNA as a template. Our method will be particularly useful for experiments requiring rapid and accurate genotyping of gene-modified animals/fetuses.

Key words: crude DNA solution, real-time PCR, SYBR Green, zygosity, transgenic mice, gene-targeted mice

Introduction

Production and maintenance of gene-engineered mice always requires genotyping. Genotyping by PCR is now widely recognized as a more effective method for identification of gene-engineered animals than traditional methods such as Southern blot and dot/slot blot hybridizations. However, this standard PCR method has some drawbacks; for example, it is difficult to distinguish heterozygous transgenic mice from homozygous transgenic ones, as well as to determine the copy number of the transgene integrated into the host genome. Recently, several groups have reported the usefulness of real-time PCR using TaqMan probes in determining the zygosity of gene-modified animals and plants with high accuracy (Tesson et al. 2002; Bubner et al. 2004; Mitrečić et al. 2005; Ji et al. 2005). More recently, this system has become simpler and cheaper than the previous one, due to the introduction of SYBR Green real-time genomic PCR reaction and development of simple mathematical calculations of zygosity determination (Tesson et al. 2002; Shitara et al. 2004; Prior et al. 2006; Haurogné et al. 2007). Unfortunately, the previous real-time PCR system employed purified genomic DNA as a template. It generally takes from 0.5 to 2 days to isolate genomic DNA, although the real-time PCR itself can be completed within 2-3 hours.

Genotyping by PCR has been completed within 2-4 hours when unpurified DNA solution is used as a template (Sakurai et al. 1995). We suspected that real-time PCR-mediated genotyping could be much simplified if crude DNA extract were used as a template.

In this study, we attempted to use crude DNA solution, which can be prepared from pieces of ear, tail, and fetus of gene-engineered mice by brief incubation (1 hour at 56^{o}C) in lysis buffer, as a template for SYBR Green real-time PCR. We found that our method is effective for rapid zygosity determination in mice.

Methods, Results, and Discussion

Preparation of crude DNA solution for real-time genomic PCR as a template

Four gene-modified mice including ROSA26 (a generous gift from Dr T. Tada of Kyoto University; Zambrowicz et al. 1997), B6ZP3Cre-Tg (purchased from The Jackson Laboratory; de Vries et al. 2000), B6rAdrenomedullin-Tg (B6rAM-Tg) (Shindo et al. 2000), and receptor activity modifying protein 2 gene-targeted mice (Ramp2KO) (Ichikawa-Shindo et al. submitted) were used in this study. Experiments were carried out in accordance with the *Guidelines of the Shinshu University Ethics Committee for Animal Experiments.*

A piece of ear (with approximately 1 mm in diameter) was obtained using an ear punch (World Precision Instruments, Inc., FL, USA). A tail fragment (approximately 1 mm in length from the tail end) was cut from each mouse. A portion (hand) of a mid-gestational fetus was collected from pregnant females 12.5 days after successful matting. Each of these samples collected in a 1.5-ml Eppendorf tube was mixed with 300 μl of lysis buffer (125 μg/ml

proteinase K, 100 mM Tris-Cl (pH 8.3), 100 mM KCl, 0.02% gelatin and 0.45% Tween 20) and incubated at 56°C for 1 hour. Samples were then boiled for 10-15 min to inactivate enzymatic activity, and spun down at 12,000 rpm for 5 min at 4°C. The supernatant (150 μl) was collected and stored at -20°C until use as crude DNA solution for real-time genomic PCR. When the crude DNA solution was diluted, TE buffer containing 50 ng/ml of yeast DNA was used.

Genotyping by PCR for mouse ROSA26 strain

PCR primers used were R26a (5'-GGCTTAAAGGCTAACCTGATGTG-3'), R26b (5'-GCGAAGAGTTTGTCCTCAACC-3'), and R26c (5'-GGAGCGGGAGAAATGGATATG-3') (Zambrowicz et al., 1997). The PCR reaction mixture (25 μl) contained 2 μl of each crude DNA solution described above, 1X PCR buffer, 1.5 mM $MgCl_2$, 300 pmol dNTPs, 12 pmol of R26a, 6 pmol of other primers, and 0.25 U rTaq DNA polymerase (Takara Bio. Co. Ltd., Kyoto, Japan). The PCR conditions were 94°C for 2 min; 33 cycles of 94°C for 30 sec, 62°C for 60 sec, and 72°C for 90 sec; and 72°C for 5 min. This primer set yields 374-bp band corresponding to the wild-type mouse, 374/1146-bp bands corresponding to the heterozygous mouse, and 1146-bp band corresponding to the homozygous mouse.

SYBR Green real-time genomic PCR and zygosity determination

Reactions were carried out on the ABI PRISM 7300 Real-Time PCR System (Applied Biosystems) using three-step cycling conditions of 95°C for 2 min, followed by 40 cycles of 95°C for 15 sec, 60°C for 30 sec, and 72°C for 1 min. PCR products were detectable in the presence of SYBR Green. The reaction mixture (25 μl) contained 2 μl of each crude DNA solution, 1x SYBR Green Real-Time PCR Master Mix (#QPK-201; TOYOBO CO. LTD., OSAKA, Japan), and 6.25 pmol of each primer. The reaction sensitivity of 2 μl of original crude DNA solution was almost equal to that of 50-100 ng of purified genomic DNA (data not shown).

For detection of β-gal gene in the ROSA26 mouse, a primer set (5'-GACCGCTGGGATCTGCCATTGTCAGACATG-3' and 5'-CCATGTGCCTTCTTCCGCGTGCAGCAGATG-3') was used. This PCR yields a band with an expected size of 204 bp. PCR using a primer set (5'-GAAGATCTTCCAATTTACTGACCGTACAC-3' and 5'-CCATGAGTGAACGAACCTGGTCGA-3') for detection of the Cre gene in the B6ZP3Cre mouse yields a band with an expected size of 450 bp. PCR with a primer set (5'-GCACGTCTAGCACCCCACA-3' and 5'-GCGGTAGCGTTTGACTCGA-3') for detection of the rat adrenomedullin (AM) gene in the B6rAM Tg mouse yields a band with expected size of 65 bp. PCR with a primer set (5'-GCTTTGGACCAGGATGTGAT-3' and 5'-CCAAACCTATCGACGTCCT-3) for detection of the deleted type of ramp2 gene in the Ramp2 KO mouse yields a band with expected size of 328 bp. A primer set (5'-GAGCCACGTGTGACCTACTGACCCACCTGC-3' and

5'-CCTGTGGGGCTAAGTCCCCTGTGAGTGACA-3') was used for detection of a 330-bp band, which corresponds to the internal (reference) ramp 3 gene.

A standard curve was prepared using 2 μl of crude DNA solutions, in which serially diluted samples (original, 2-, 4-, 8-, 16-diluted) were included. The slopes of Ct and dCt ((target gene)-(reference gene)) and R2 values of each sample were calculated by the ABI PRISM 7300 SDS vl.X and Microsoft Excel 2004 for Mac ver. 11.35. Relative quantification of zygosity was performed with the 2^{-ddCt} method (Livak and Schmittgen, 2001).

Actual trial of zygosity determination using ROSA26 strain

The ROSA26 mouse strain was used as a model to test whether the SYBR Green real-time PCR system enables reporting between two–fold differences in zygosity. In this case, β-gal and ramp3 genes were amplified as target and reference genes, respectively. First, a crude DNA solution prepared from an ear piece of heterozygous or homozygous ROSA26 mouse was serially diluted and a total of 5 solutions (original, 2-, 4-, 8-, 16-dilution) were subjected to PCR as a reaction template. We employed the 3-step reaction-mediated amplification for determination of zygosity. The standard curves obtained are depicted in Figure 1A. The slopes of these standard curves indicated that amplification efficiency was approximately 99%, and the efficiencies of target and reference were almost equal. These results indicate that i) PCRs for both target and reference genes exhibit similar efficiency, and ii) the 2^{-ddCt} method is adequate for evaluation of this system.

Next, we performed SYBR Green real-time genomic PCR using the same conditions as described above for five serially diluted crude DNA samples (original, 2-, 4-, 8-, 16-dilution) prepared from the tail and hand of the 12.5dpc fetus (Figures 1B and 1C). The patterns of curves were quite similar to those of curves obtained from ear sample (Figure 1A), suggesting that the crude DNA solution prepared from several tissues can be used for SYBR Green real-time genomic PCR as an alternative to the purified DNA. In fact, we tested use of ear samples from mice (1-month-old) obtained after mating between heterozygous ROSA26 mice, and genotype-known samples (as controls), so-called K1 (heterozygous sample) and K2 (homozygous sample). The value of 2^{-ddCt} was calculated from the dCt (β-gal -ramp3) of each sample, together with the dCt of the K1 sample (as a calibrator). Representative findings are shown in Table 1. The 2^{-ddCt} value of the K2 sample was 2.1, coincident with the expected value of 2. The 2^{-ddCt} values of sample Nos. 1-6 listed in Table 1 were 1.06, 1.28, 0.08, 2.01, 1.33, and 0.11, which were judged to be heterozygous, heterozygous, wild-type, homozygous, heterozygous, and wild-type, respectively. The accuracy of these results was confirmed by another PCR-mediated genotyping method for ROSA26 (Table 1; electrophoretic data not shown; see *Genotyping by PCR for mouse ROSA26 strain*). The present method employing crude DNA extract as a template is thus useful for distinguishing between two-fold differences in zygosity.

Zygosity determination of several gene-modified strains

We examined whether the present method is applicable to determination of zygosity in other gene-modified mouse strains such a transgenic B6ZP3Cre-Tg (carrying Cre gene linked to the zona pellucida 3 promoter), transgenic B6rAM-Tg (carrying rat adrenomedullin cDNA linked to the αMHC promoter), and the recently established KO line Ramp2 KO (Ichikawa-Shindo et al., submitted). When the same amplification procedure as applied to ROSA26 was employed, the amplification efficiencies of target and reference genes were almost equal (data not shown). For real-time PCR analysis of B6ZP3Cre-Tg, the target and reference genes were Cre and ramp3 genes, respectively. Ears of one-month-old mice obtained from mating between heterozygous females and homozygous males and from genotype-known samples were used as a control. For real-time PCR analysis of B6rAM-Tg, the target and reference genes were rat AM and mouse ramp3 genes, respectively. The hand of 12.5-dpc fetus, obtained from mating between heterozygous mice, was amplified together with genotype-known control samples. Finally, for real-time PCR analysis of Ramp2 KO, target and reference genes were the deleted-type of ramp2 and ramp3 genes, respectively. The hand of 12.5-dpc fetuses, obtained from mating between heterozygous females and homozygous males, was amplified together with genotype-known control samples. In each case, a sharp distinction in zygosity was observed. The data obtained from real-time PCR of these samples are summarized in Table 1.

Effectiveness of SYBR Green real-time genomic PCR using a crude DNA template

In this study, we show that crude DNA extracts can be used as a good template for SYBR Green real-time genomic PCR. The biggest advantage of this system appears to be improvement of the time required for zygosity determination, since only one hour is needed to acquire a crude DNA sample. This is in contrast to the previous system, which depends on the use of purified DNA and which generally requires for 0.5-2 days for processing. The present method will be particular importance when rapid manipulation of a fetus derived from complex mating between transgenics and KO mice required for exploration of biological functions of isolated fetal cells or tissues.

In conclusion, our real-time PCR method employing crude DNA extract as a template was proven effective for zygosity determination in gene-engineered mice. The method appears to be more convenient, cost-effective, and rapid than the previous amplification system, which employs purified DNA as a template.

Acknowledgement

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References

Bubner B, Gase K, Baldwin IT (2004) Two-fold differences are the detection limit for determining transgene copy numbers in plants by real-time PCR. BMC Biotechnol 4:14

de Vries WN, Binns LT, Fancher KS, Dean J, Moore R, Kemler R, Knowles BB (2000) Expression of Cre recombinase in mouse oocytes: a means to study maternal effect genes. Genesis 26:110-112

Haurogné K, Bach JM, Lieubeau B (2007) Easy and rapid method of zygosity determination in transgenic mice by SYBR Green real-time quantitative PCR with a simple data analysis. Transgenic Res 16:127-131

Ji W, Zhou W, Abruzzese R, Guo W, Blake A, Davis S, Davis S, Polejaeva I (2005) A method for determining zygosity of transgenic zebrafish by TaqMan real-time PCR. Anal Biochem 344:240-246

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method 25:402-408

Mitrečić D, Huzak M, Ćurlin M, Gajović S (2005) An improved method for determination of gene copy numbers in transgenic mice by serial dilution curves obtained by real-time quantitative PCR assay. J Biochem Biophys Methods 64:83-98

Prior FA, Tackaberry ES, Aubin RA, Casley WL (2006) Accurate determination of zygosity in transgenic rice by real-time PCR does not require standard curves or efficiency correction. Transgenic Res 15:261-265

Sakurai T, Iguchi T, Moriwaki K, Noguchi M (1995) The ter mutation first causes primordial germ cell deficiency in ter/ter mouse embryos at 8 days of gestation. Dev Growth Differ. 37:293-302

Shindo T, Kurihara H, Maemura K, Kurihara Y, Kuwaki T, Izumida T, Minamino N, Ju KH, Morita H, Oh-hashi Y, Kumada M, Kangawa K, Nagai R, Yazaki Y (2000) Hypotension and resistance to lipopolysaccharide-induced shock in transgenic mice overexpressing adrenomedullin in their vasculature. Circulation 101:2309-2316.

Shitara H, Sato A, Hayashi J, Mizushima N, Yonekawa H, Taya C (2004) Simple method of zygosity identification in transgenic mice by real-time quantitative PCR. Transgenic Res 13:191-194

Tesson L, Heslan JM, Ménoret S, Anegon I (2002) Rapid and accurate determination of zygosity in transgenic animals by real-time quantitative PCR. Transgenic Res 11:43-48

Zambrowicz BP, Imamoto A, Fiering S, Herzenberg LA, Kerr WG, Soriano P (1997) Disruption of overlapping transcripts in the ROSA beta geo 26 gene trap strain leads to widespread expression of beta-galactosidase in mouse embryos and hematopoietic cells. Proc Natl Acad Sci U S A. 94:3789-3794

Figure legends

Figure 1. Efficiency of amplification of β-gal and ramp3 obtained by SYBR Green real-time genomic PCR of crude DNA prepared from ear (A), tail (B), and fetus (C) of ROSA26 mouse. On the right side, data from homozygous samples are shown, while on the left side data from heterozygous samples are shown. Each ordinate unit is a log scale of a dilution series (original=1, 2-fold dilution=1/2, 4-fold dilution=1/4, 8-fold dilution=1/8, and 16-fold dilution=1/16) of crude DNA solution. The unit of each abscissa is the number of cycle amplifications (Ct). Results are representatives of 3-5 experiments. Note that each curve of β-gal and ramp3 exhibited a similar pattern of slope, indicating that PCR for target and reference genes exhibits similar efficiency, and that the 2^{-ddCt} method is adequate for evaluation of this system. Furthermore, spacing between the two curves for heterozygous sample was approximately 1/2 that for homozygous one. The slopes of Ct and dCt ((β-gal)-(ramp3)) and R2 values of each sample were calculated by the ABI PRISM 7300 SDS and Microsoft Excel.

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Table 1 Representative data for zygosity determination with clude DNA solutions derived from several gene-modified mouse strain

Strain	Crude DNA solution		K1	K2	Samples					
			Heterozygous	Homozygous	1	2	3	4	5	6
ROSA26	ear	dCt (β-gal -ramp3)	1.98	0.92	1.9	1.63	5.55	0.98	1.58	5.2
		ddCt(dCt(Sample)-dCt(K1))	0	-1.07	-0.09	-0.36	3.56	-1.01	-0.41	3.21
		2^{-ddCt}	1	2.1	1.06	1.28	0.08	2.01	1.33	0.11
		Expected zygosity	β/+	β/β	β/+	β/+	wild	β/β	β/+	wild
		Zygosity detemination by electrophretic analysis [a]	β/+	β/β	β/+	β/+	wild	β/β	β/+	wild
ZP3Cre-Tg	ear	dCt (Cre -ramp3)	-5.58	-6.52	-5.56	-6.61	-6.75	-5.61	-5.64	-6.5
		ddCt(dCt(Sample)-dCt(K1))	0	-0.94	0.02	-1.03	-1.17	-0.03	-0.07	-0.92
		2^{-ddCt}	1	1.92	0.99	2.04	2.25	1.02	1.05	1.89
		Expected zygosity	Cre/+	Cre/Cre	Cre/+	Cre/Cre	Cre/Cre	Cre/+	Cre/+	Cre/Cre
rAM-Tg	embryo	dCt (rAM -ramp3)	0.84	0.05	0.64	3.02	-0.19	0.53	2.84	0.66
		ddCt(dCt(Sample)-dCt(K1))	0	-0.79	-0.2	2.18	-1.03	-0.32	2.01	-0.18
		2^{-ddCt}	1	1.73	1.15	0.22	2.04	1.25	0.25	1.14
		Expected zygosity	rAM/+	rAM/rAM	rAM/+	wild	rAM/rAM	rAM/+	wild	rAM/+
Ramp2 KO	embryo	dCt (ramp2ko -ramp3)	2.94	2.05	4.9	4.73	2.6	1.87	2.8	3.06
		ddCt(dCt(Sample)-dCt(K1))	0	-0.89	1.96	1.79	-0.34	-1.07	-0.14	0.12
		2^{-ddCt}	1	1.85	0.26	0.29	1.26	2.1	1.1	0.92
		Expected zygosity	KO/+	KO/KO	wild	wild	KO/+	KO/KO	KO/+	KO/+

β, β-gal gene; wild, wild-type

[a] Accuracy of results was confirmed by another PCR-mediated genotyping method for ROSA26 (see *Genotyping by PCR for mouse ROSA26 strain*). PCR products were analyzed by electrophoresis, with 374-bp band, 374/1146-bp bands, and 1146-bp band regarded as wild-type, heterozygous, and homozygous genotype, respectively.

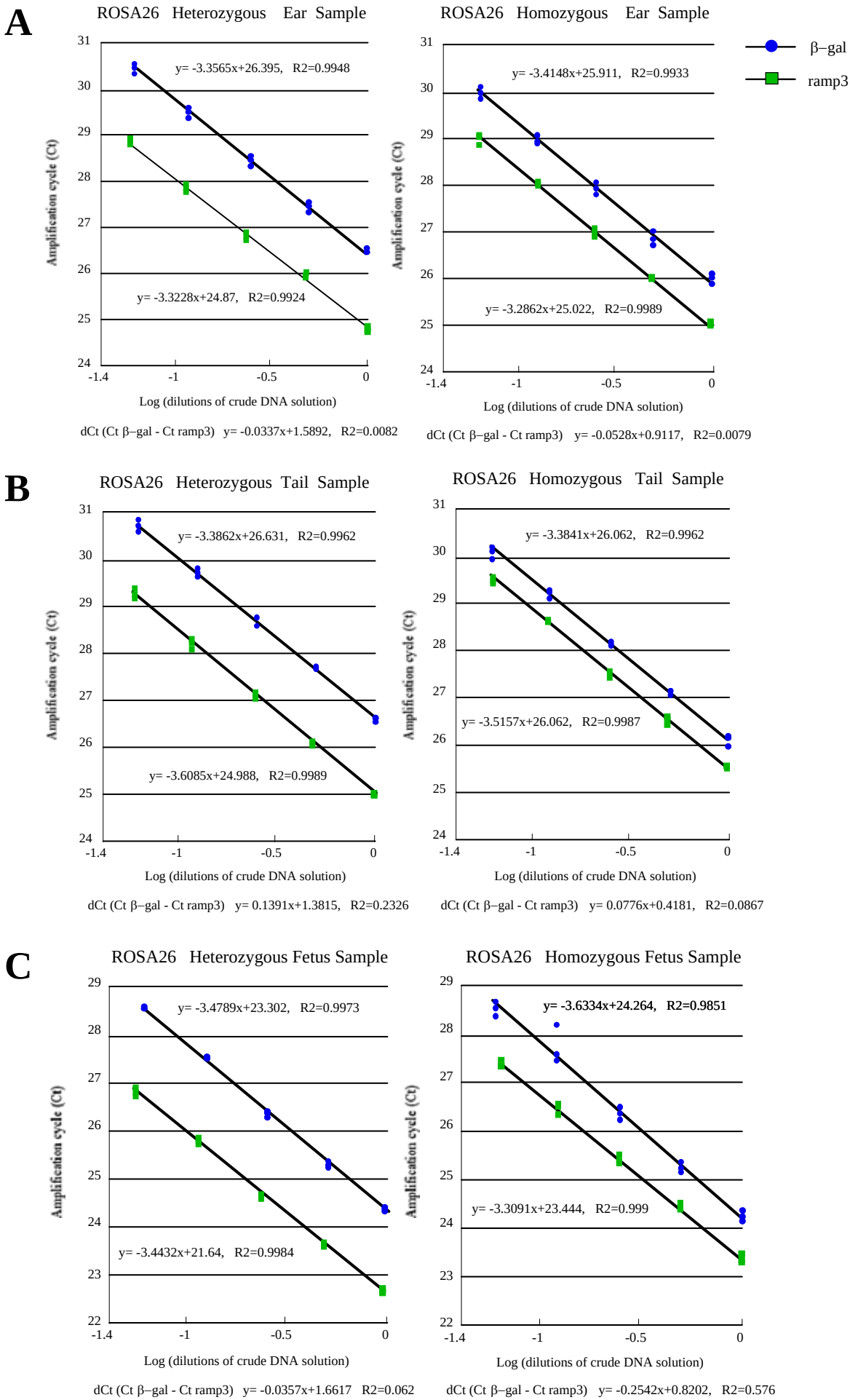
A
ROSA26 Heterozygous Ear Sample
y= -3.3565x+26.395, R2=0.9948
y= -3.3228x+24.87, R2=0.9924
Amplification cycle (Ct)
Log (dilutions of crude DNA solution)
dCt (Ct β–gal - Ct ramp3) y= -0.0337x+1.5892, R2=0.0082
ROSA26 Homozygous Ear Sample
y= -3.4148x+25.911, R2=0.9933
y= -3.2862x+25.022, R2=0.9989
Log (dilutions of crude DNA solution)
dCt (Ct β–gal - Ct ramp3) y= -0.0528x+0.9117, R2=0.0079
β–gal
ramp3
B
ROSA26 Heterozygous Tail Sample
y= -3.3862x+26.631, R2=0.9962
y= -3.6085x+24.988, R2=0.9989
Log (dilutions of crude DNA solution)
dCt (Ct β–gal - Ct ramp3) y= 0.1391x+1.3815, R2=0.2326
ROSA26 Homozygous Tail Sample
y= -3.3841x+26.062, R2=0.9962
y= -3.5157x+26.062, R2=0.9987
Log (dilutions of crude DNA solution)
dCt (Ct β–gal - Ct ramp3) y= 0.0776x+0.4181, R2=0.0867
C
ROSA26 Heterozygous Fetus Sample
y= -3.4789x+23.302, R2=0.9973
y= -3.4432x+21.64, R2=0.9984
Log (dilutions of crude DNA solution)
dCt (Ct β–gal - Ct ramp3) y= -0.0357x+1.6617 R2=0.062
ROSA26 Homozygous Fetus Sample
y= -3.6334x+24.264, R2=0.9851
y= -3.3091x+23.444, R2=0.999
Log (dilutions of crude DNA solution)
dCt (Ct β–gal - Ct ramp3) y= -0.2542x+0.8202, R2=0.576

Table 1 Representative data for zygosity determination with clude DNA solutions derived from several gene-modified mouse strain

Strain	Crude DNA solution.		K1	K2	Samples					
			Heterozygous	Homozygous	1	2	3	4	5	6
ROSA26	ear	dCt (β-gal-ramp3)	1.98	0.92	1.9	1.63	5.55	0.98	1.58	5.2
		ddCt(dCt(Sample)-dCt(K1))	0	-1.07	-0.09	-0.36	3.56	-1.01	-0.41	3.21
		2^{-ddCt}	1	2.1	1.06	1.28	0.08	2.01	1.33	0.11
		Expected zygosity	β/+	β / β	β/+	β/+	wild	β / β	β/+	wild
		Zygosity determination by electrophoretic analysis [1]	β/+	β / β	β/+	β/+	wild	β / β	β/+	wild
ZP3Cre-Tg	ear	ddCt(dCt(Sample)-dCt(K1))	0	-0.94	0.02	-1.03	-1.17	-0.03	-0.07	-0.92
		2^{-ddCt}	1	1.92	0.99	2.04	2.25	1.02	1.05	1.89
		Expected zygosity	Cre/+	Cre/Cre	Cre/+	Cre/Cre	Cre/Cre	Cre/+	Cre/+	Cre/Cre
rAM-Tg	fetus	dCt (rAM -ramp3)	0.84	0.05	0.64	3.02	-0.19	0.53	2.84	0.66
		ddCt(dCt(Sample)-dCt(K1))	0	-0.79	-0.2	2.18	-1.03	-0.32	2.01	-0.18
		2^{-ddCt}	1	1.73	1.15	0.22	2.04	1.25	0.25	1.14
		Expected zygosity	rAM/+	rAM/rAM	rAM/+	wild	rAM/rAM	rAM/+	wild	rAM/+
Ramp2KO	fetus	dCt (ramp2KO -ramp3)	2.94	2.05	4.9	4.73	2.6	1.87	2.8	3.06
		ddCt(dCt(Sample)-dCt(K1))	0	-0.89	1.96	1.79	-0.34	-1.07	-0.14	0.12
		2^{-ddCt}	1	1.85	0.26	0.29	1.26	2.1	1.1	0.92
		Expected zygosity	KO/+	KO/KO	wild	wild	KO/+	KO/KO	KO/+	KO/+

β, β-gal gene; wild, wild-type

1. Accuracy of results obtained was confirmed by another PCR-mediated genotyping method for ROSA26 (see *Genotyping by PCR for mouse ROSA26 strain*). PCR products were analyzed by electrophoresis, with 374-bp band, 374/1147-bp bands, and 1147-bp band regarded as w ild-type, heterozygous, and homozygous genotype, respectively.