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Simultaneous Detection of Multiple Transgenes for Genetically-Modified Mouse Strains

Hatsumi NAKATA, Tomomi HASHIMOTO, Yukiko SEKI,
Kazuyuki MEKADA, Yuichi OBATA, and Atsushi YOSHIKI

*Experimental Animal Division, RIKEN BioResource Center,
3-1-1 Koyadai, Tsukuba, Ibaraki 305-0074, Japan*

Abstract: Recent advances in the genetic manipulation of mice have enabled us to generate transgenic and knockout mice. However, it is not easy to maintain these genetically-modified mice with the high-quality necessary to meet both scientific and legal requirements. RIKEN BRC has collected various transgenic, knockout, and conditional knockout mice. To examine the genetic modifications in these strains quickly and thoroughly, we established a simultaneous PCR test to detect multiple transgenes. We have called this, the “KO-survey”. The PCR condition was optimized to detect *neo*, *puro*, *pgk-neo*, *lacZ*, and *HSVtk-neo* in set I, and *hyg*, *IREs*, *cre*, *flp*, and *Gfp* in set II. This “KO-survey” is useful for providing users with mouse strains of the highest genetic quality and accurate information on their genetic modifications.

Key words: Cartagena Law, genotyping, knockout mice

Mice are one of the most important animal models in life sciences [2, 6, 26]. Recent advances in the genetic manipulation of mice using various technologies and systems have enabled us to generate transgenic and knockout mice with precise control of the gene expression [4, 19, 25]. The recombinant gene constructs designed for genetic modifications are becoming increasingly complex with multiple components, such as promoters, selectable markers, visualizing reporters, recombinase genes, and their target sequences [1, 15, 18, 19]. Recently, multiple crosses of transgenic and knockout mice have become common procedures in the functional analyses [16, 18, 23]. Therefore, it has become necessary for a mouse resource center to thoroughly examine these multiple transgenes, whether or not they

are kept as originally designed, and when new strains are deposited.

RIKEN BioResource Center (BRC) was established in 2001 as a comprehensive biological resource center in Japan. Our primary mission is to collect, preserve, conduct quality control, and distribute mouse strains developed mainly by Japanese scientists. We have been acting as the core facility of the mouse resources in the National BioResource Project (NBRP) promoted by the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan since 2002. The mouse strain collection includes various genetically-modified strains such as transgenic, knockout, and conditional knockout mice [26].

In Japan, all handling and activity with genetically-

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Address corresponding: A. Yoshiki, Experimental Animal Division, RIKEN BioResource Center, 3-1-1 Koyadai, Tsukuba, Ibaraki 305-0074, Japan

Table 1. Various transgenes used in the genetically-modified mouse strains at the RIKEN BRC according to depositor's information provision

Name of the transgene	Purpose/Function	Species origin	Number of transgenes in		Total	%
			Knockout mice	Transgenic mice		
neomycin resistance gene (<i>neo</i>)	Drug-resistance selection marker	<i>E. coli</i>	285	6	291	28.6
Green fluorescence protein (<i>Gfp</i>)	Fluorescent reporter	Jellyfish	21	208	229	22.5
phosphoglycerate kinase and neomycin resistance fusion gene cassette (<i>pgk-neo</i>)	Promoter and selection marker	Mouse and <i>E. coli</i>	168	4	172	16.9
beta galactosidase gene (<i>lacZ</i>)	Histochemical reporter	<i>E. coli</i>	108	60	168	16.5
Cre recombinase gene (<i>cre</i>)	Recombinase for conditional knockout	Phage P1	16	44	60	5.9
HSV thymidine kinase and neomycin resistance fusion gene cassette (<i>HSVtk-neo</i>)	Promoter and selection marker	Herpes simplex virus (HSV) and <i>E. coli</i>	46	0	46	4.5
Internal Ribosome Entry Site (<i>IRES</i>)	Internal Ribosome Entry Site	Encephalomyocarditis virus (EMCV)	18	21	39	3.8
puromycin resistance gene (<i>puro</i>)	Drug-resistance selection marker	<i>Streptomyces alboniger</i>	1	5	6	0.6
Flipase gene (<i>flp</i>)	Recombinase for conditional knockout	Yeast	1	4	5	0.5
hygromycin resistance gene (<i>hyg</i>)	Drug-resistance selection marker	<i>E. coli</i>	3	0	3	0.3
Total number of transgenes			667	352	1,019	100.0
Number of strains			512	310	822	

modified animals must be conducted in compliance with all applicable laws, including the Law Concerning the Conservation and Sustainable Use of Biological Diversity Through Regulations on the Use of Living Modified Organisms, the so-called “Cartagena Law”. Under this law a scientist and her/his organization must provide accurate information on the genetic modification when they transport genetically-modified mice to another organization. To continue operation of the NBRP core facility of mouse resources, we need to thoroughly examine the genetic modifications of the genetically-modified strains and maintain the highest genetic quality that meets both the scientific and legal requirements. For this purpose, we have recently established a simultaneous detection PCR test for multiple transgenes, designated the “KO-survey”, as part of our genetic quality control program.

For the development of the KO-survey, first we examined the information provided from the depositor scientists of 822 genetically-modified (512 knockout and 310 transgenic) strains carrying 1,019 transgenes. As listed in Table 1, we have selected ten representative transgenes, *neo*, *Gfp*, *pgk-neo*, *lacZ*, *cre*, *HSVtk-neo*, *IRES*, *puro*, *flp*, and *hyg*, to be examined in the KO-survey PCR. The number of each transgene in the knockout and transgenic strains was counted and its frequency expressed as a percentage (%). The use of *puro*, *flp*, and *hyg* is expected to increase in the near future because of an anticipated rapid increase in the generation of conditional knockout mice [5], and they were included in the KO-survey PCR, even though they are currently minor components with frequencies of less than 1%. The CAG-promoter is a widely used as a transgenic component [20], but is not included in Table 1. Transgenes with

Table 2. List of primers for the KO-survey PCR

Set name	Gene name	Primer sequence (upper; forward primer, lower; reverse primer)	Product size	GenBank accession No.	References
Set I	<i>neo</i>	5'-TGGATTGCACGCAGGTCTC-3' 5'-CGGCCACAGTCGATGAATCC-3'	617 bp	NC_008460	[7]
	<i>puro</i>	5'-ATGACCGAGTACAAGCCAC-3' 5'-GGCACCCCGGCGCGCTCGGC-3'	470 bp	M25346	[3], http://www.jax.org/ This study
	<i>pgk-neo*</i>	5'-CGCGCCACCTTCTACTCCTC-3' 5'-GCAATCCATCTTGTTCAATGGCC-3'	393 bp	NT_039706 (<i>pgk</i>) AF335419 (<i>pgk-neo</i>)	This study [11]
	<i>lacZ</i>	5'-ATCCTCTGCATGGTCAGGTC-3' 5'-CGTGGCCTGATTCATTCC-3'	315 bp	NC_011750	[3], http://www.jax.org/
	<i>HSVtk-neo*</i>	5'-CAAACCCCGCCCAGCGTCTTGTC-3' 5'-GCAATCCATCTTGTTCAATGGCC-3'	163 bp	X14112 (<i>HSVtk</i>) U43612 (<i>HSVtk-neo</i>)	This study [11]
Set II	<i>hyg</i>	5'-GCCTGACCTATTGCATCTCC-3' 5'-CGGCGAGTACTTCTACACAG-3'	723 bp	V01499	This study
	<i>IRES**</i>	5'-gggatccgCCCCTCTCCCTCCCCCCCCCTAACG-3' 5'-gagatcTGTGGCCATATTATCATCGTGTTC-3'	597 bp	X74312	This study
	<i>cre</i>	5'-TCGATGCAACGAGTGATGAG-3' 5'-TTCGGCTATACGTAACAGGG-3'	482 bp	NC_005856	[10]
	<i>flp</i>	5'-CTGCTTCTTCCGATGATTCG-3' 5'-CACCACCTAAGGTGCTTGTC-3'	372 bp	U46493	[9]
	<i>Gfp</i>	5'-AAGTTCATCTGCACCACCG-3' 5'-TCCTGAAGAAGATGGTGCG-3'	173 bp	U55763	[3], http://www.jax.org/

*Forward primers for *pgk-neo* and *HSVtk-neo* were specific to mouse *pgk* and *HSVtk*, respectively. Reverse primers for *pgk-neo* and *HSVtk-neo* were common and specific to *neo*. Therefore, the primer set for *pgk-neo* does not amplify the endogenous mouse *pgk*. **Primers for *IRES* contain additional sequences as indicated by small letters for cloning and adjustment of the product size (597 bp) to make it appropriate for the KO-survey PCR.

CAG-promoter are usually integrated as multiple transgenes in a tandem arrangement, and may result in multiple PCR fragments of various sizes (data not shown). Therefore, we detect the transgenes with CAG-promoter separately in a single PCR test, not included in the KO-survey PCR.

Next, we tried to set up a multiplex PCR that simultaneously detects the ten transgenes in minimal reactions. Primer sequences were retrieved from public databases [3], other publications [7, 9, 11] or were originally designed for this study (Table 2). The following mouse strains carrying specific transgenes were used to extract genomic DNA for the optimization of the multiplex PCR: B6.Cg-TgTn(*sb-lacZ*,*GFP*)*IF2J*tk (RBRC01652) carrying *neo*, *lacZ*, *pgk-neo*, *IRES* and *Gfp* [14], C57BL/6-Tg(*CAG-FLPe*)36*Ito* (RBRC01834) carrying *puro* [13], CNR CP carrying *HSVtk-neo* (an unpublished strain kindly provided by Dr. T. Yagi, Osaka University), B6.129P2-*Emx1*^{tm2(*cre*)*llo*} (RBRC01345) carrying *cre* [12],

C57BL/6N-Tg(*EIF1A-flp*)66*Mim* (RBRC01252) carrying *flp* [24], FVB.129P2-*Nf2*^{tm1*Gih*} (RBRC01678) carrying *hyg* [10], and C57BL/6Jcl (CLEA Japan, Inc., Tokyo, Japan) for wild-type DNA. Genomic DNAs were extracted from tail tips by automated DNA isolators (NA-2000 and PI-200, Kurabo, Kyutaro-machi, Osaka, Japan) with a final concentration of 100–300 ng/ μ l. Three microliters of genomic DNA was used as the template. A mixture solution of the same volume of genomic DNA from the above genetically-modified strains was used as a positive control for the KO-survey PCR.

It was found that two sets of multiplex PCR reactions are enough to survey all the transgenes. We optimized the PCR conditions with a Biometra Thermocycler (Goettingen, Germany) to simultaneously amplify five transgenes in one tube, such as *neo*, *puro*, *pgk-neo*, *lacZ*, and *HSVtk-neo* in set I using LA-Taq with GC-I buffer (Takara, Kyoto, Japan), and *hyg*, *IRES*, *cre*, *flp*, and *Gfp* in set II using Multiplex PCR Kit (Qiagen, Hilden, Ger-

Table 3. Components of reaction mixture for the KO-survey PCR

Set I (Total volume 20 μ l)		Set II (Total volume 20 μ l)	
2 $\times$ GC-I Buffer	10.0 μ l	2 $\times$ Master Mix	10.0 μ l
2.5 mM dNTP	2.0 μ l	Distilled water	6.0 μ l
Distilled water	3.8 μ l	Mixed primers solution*	1.0 μ l
Mixed primers solution*	1.0 μ l	<i>hyg</i>	15 μ M, 15 μ M
<i>neo</i>	5 μ M, 5 μ M	<i>IRES</i>	5 μ M, 5 μ M
<i>puro</i>	2.5 μ M, 2.5 μ M	<i>cre</i>	5 μ M, 5 μ M
<i>pgk-neo</i>	10 μ M, 10 μ M	<i>flp</i>	15 μ M, 15 μ M
<i>lacZ</i>	15 μ M, 15 μ M	<i>Gfp</i>	10 μ M, 10 μ M
<i>HSVtk-neo</i>	20 μ M, 15 μ M	Template DNA	3.0 μ l
Template DNA	3.0 μ l		
LA Taq (5 units/ μ l)	0.2 μ l		

*Each mixed primer solution contains forward and reverse primers of each transgene. The final concentration of each primer (forward, reverse) is indicated.

Fig. 1. Results of KO-survey PCR using positive control DNA. (A) *neo*, *puro*, *pgk-neo*, *lacZ*, and *HSVtk-neo*: Each transgene was amplified with a single primer set by using a positive control (P) and C57BL/6J DNA (N). Set I: Multiple transgenes were amplified simultaneously by the KO-survey PCR set I using a positive control (P) and C57BL/6J DNA (N). Lanes M: DNA Marker (100 bp ladder; Invitrogen Japan K.K., Tokyo). (B) *hyg*, *IRES*, *cre*, *flp*, and *Gfp*: Each transgene was amplified with a single primer set by using a positive control (P) and C57BL/6J DNA (N). Set II: Multiple transgenes were amplified simultaneously by the KO-survey PCR set II using a positive control (P) and C57BL/6J DNA (N). Lanes M: DNA Marker (100 bp ladder).

many) (Table 3). The PCR temperatures and cycling for sets I and II are as follows: set I has an initial heating at 94°C for 180 s, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 60 s, and final extension at 72°C for 300 s; and set II has an initial heating at 95°C for 900 s, followed

by 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 90 s, extension at 72°C for 90 s, and final extension at 72°C for 600 s. The PCR products are electrophoresed in 2% agarose gels using 1 $\times$ TAE buffer, and stained with SYBR Safe DNA Gel stain (Invitrogen Japan K. K., Tokyo, Japan). The gels are photographed

Fig. 2. Detection of *pgk-neo* and *cre* in conditional knockout mice by the KO-survey PCR. (A) Heterozygous ICR.Cg-Ripply2^{tm1.Ysa} mice (*flox/+*, Nos. 1 and 2) carried a floxed allele containing a *pgk-neo* cassette. (B) Heterozygous Ripply2^{tm1.Ysa/+} (*delta neo/+*) descendants (Nos. 3 and 4) after crossing a heterozygous ICR.Cg-Ripply2^{tm1.Ysa} mouse with a CAG-cre mouse to delete the floxed *pgk-neo* in the Ripply2 [17]. Lanes M, P, and N indicate DNA Marker (100 bp ladder), and positive and negative controls.

under UV light. The PCR products of the predicted sizes are shown as Figs. 1A and 1B.

Using the KO-survey PCR we screened 771 genetically-modified strains deposited at RIKEN BRC. The *pgk-neo* and HSVtk-*neo* promoter-selection marker gene cassettes were detected in 72 (9.3%) and 32 (4.2%) strains, respectively, the information for which did not contain *pgk* and HSVtk promoters. The following transgenes were not stipulated in the provided information, and were detected unexpectedly: *cre* and *flp* recombinase genes in 24 strains (3.1%), *Gfp*, *lacZ*, and other visualizing reporters in 9 strains (1.2%). A total of 137 out of 771 (17.8%) strains were found to have insufficient information about their genetic modifications, or had not been thoroughly examined in the course of establishing the strains by crossing with Cre-driver or GFP-reporter mice. Their genetic quality and relevant information must be improved prior to reproduction, preservation at our facility and distribution to users.

A typical example of detecting *pgk-neo* and *cre* in conditional knockout mice using the KO-survey PCR is shown in Fig. 2. Heterozygous ICR.Cg-Ripply2^{tm1.Ysa} (*flox/+*) mice (Nos. 1 and 2, Fig. 2A) carried a floxed *pgk-neo* cassette in the Ripply2 [17]. We could detect *cre* in one of two descendants (Nos. 3 and 4, Fig. 2B) of heterozygous Ripply2^{tm1.Ysa/+} (*delta neo/+*) mice after deleting *pgk-neo* by crossing with a CAG-cre transgenic mice [20]. To establish a strain carrying Ripply2^{tm1.Ysa} (STOCK Ripply2^{tm1.Ysa}), the No. 3 mouse

without *cre* was used for successive breeding, and the No. 4 mouse carrying *cre* was immediately eliminated from the colony.

Currently, a large number of reporter-null and conditional knockout mice are generated in international collaborations [6]. Multiple crosses among conditional knockout, tissue-specific Cre-driver and reporter strains are expected to increase tremendously in future functional genomics studies [5, 8, 22]. Consequently, RIKEN BRC as a dissemination center of mouse resources continues to pay much attention to the control of our genetic quality at every step from deposition, preservation and distribution of the strains. Depositor scientists are also requested to carefully screen every transgene in their genetically-modified strains prior to depositing the mice at RIKEN BRC.

In conclusion, the KO-survey PCR protocol enables us to cost-effectively screen 5 genes in one PCR tube, and to provide our users with mouse strains of the highest quality together with accurate information on their genetic modifications.

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References

1. Branda, C.S. and Dymecki, S.M. 2004. *Dev. Cell* 6: 7–28.
2. Brown, S.D., Hancock, J.M., and Gates, H. 2006. *PLoS Genet.* 2: e118.
3. Bult, C.J., Blake, J.A., Richardson, J.E., Kadin, J.A., Eppig, J.T., Baldarelli, R.M., Barsanti, K., Baya, M., Beal, J.S., Boddy, W.J., Bradt, D.W., Burkart, D.L., Butler, N.E., Campbell, J., Corey, R., Corbani, L.E., Cousins, S., Dene, H., Drabkin, H.J., Frazer, K., Garippa, D.M., Glass, L.H., Goldsmith, C.W., Grant, P.L., King, B.L., Lennon-Pierce, M., Lewis, J., Lu, I., Lutz, C.M., Maltais, L.J., McKenzie, L.M., Miers, D., Modrusan, D., Ni, L., Ormsby, J.E., Qi, D., Ramachandran, S., Reddy, T.B., Reed, D.J., Sinclair, R., Shaw, D.R., Smith, C.L., Szauter, P., Taylor, B., Vanden Borre, P., Walker, M., Washburn, L., Witham, I., Winslow, J., and Zhu, Y. 2004. *Nucleic Acids Res.* 32: D476–481.
4. Capecchi, M.R. 1989. *Science* 244: 1288–1292.
5. Collins, F.S., Finnell, R.H., Rossant, J., and Wurst, W. 2007. *Cell* 129: 235.
6. Collins, F.S., Rossant, J., and Wurst, W. 2007. *Cell* 128: 9–13.
7. Datsenko, K.A. and Wanner, B.L. 2000. *Proc. Natl. Acad. Sci. U.S.A.* 97: 6640–6645.
8. Friedel, R.H., Seisenberger, C., Kaloff, C., and Wurst, W. 2007. *Brief Funct. Genomic. Proteomic.* 6: 180–185.
9. Furuta, Y., Lagutin, O., Hogan, B.L., and Oliver, G.C. 2000. *Genesis* 26: 130–132.
10. Giovannini, M., Robanus-Maandag, E., van der Valk, M., Niwa-Kawakita, M., Abramowski, V., Goutebroze, L., Woodruff, J.M., Berns, A., and Thomas, G. 2000. *Genes Dev.* 14: 1617–1630.
11. Han, H., Tanigaki, K., Yamamoto, N., Kuroda, K., Yoshimoto, M., Nakahata, T., Ikuta, K., and Honjo, T. 2002. *Int. Immunol.* 14: 637–645.
12. Iwasato, T., Datwani, A., Wolf, A.M., Nishiyama, H., Taguchi, Y., Tonegawa, S., Knopfel, T., Erzurumlu, R.S., and Itohara, S. 2000. *Nature* 406: 726–731.
13. Kanki, H., Suzuki, H., and Itohara, S. 2006. *Exp. Anim.* 55: 137–141.
14. Keng, V.W., Yae, K., Hayakawa, T., Mizuno, S., Uno, Y., Yusa, K., Kokubu, C., Kinoshita, T., Akagi, K., Jenkins, N.A., Copeland, N.G., Horie, K., and Takeda, J. 2005. *Nat. Methods* 2: 763–769.
15. Matsumura, H., Hasuwa, H., Inoue, N., Ikawa, M., and Okabe, M. 2004. *Biochem. Biophys. Res. Commun.* 321: 275–279.
16. McHugh, T.J., Jones, M.W., Quinn, J.J., Balthasar, N., Coppari, R., Elmquist, J.K., Lowell, B.B., Faselow, M.S., Wilson, M.A., and Tonegawa, S. 2007. *Science* 317: 94–99.
17. Morimoto, M., Sasaki, N., Oginuma, M., Kiso, M., Igarashi, K., Aizaki, K., Kanno, J., and Saga, Y. 2007. *Development* 134: 1561–1569.
18. Novak, A., Guo, C., Yang, W., Nagy, A., and Lobe, C.G. 2000. *Genesis* 28: 147–155.
19. Okabe, M., Ikawa, M., Kominami, K., Nakanishi, T., and Nishimune, Y. 1997. *FEBS Lett.* 407: 313–319.
20. Sakai, K. and Miyazaki, J. 1997. *Biochem. Biophys. Res. Commun.* 237: 318–324.
21. Sakaue-Sawano, A., Kurokawa, H., Morimura, T., Hanyu, A., Hama, H., Osawa, H., Kashiwagi, S., Fukami, K., Miyata, T., Miyoshi, H., Imamura, T., Ogawa, M., Masai, H., and Miyawaki, A. 2008. *Cell* 132: 487–498.
22. Schnutgen, F., De-Zolt, S., Van Sloun, P., Hollatz, M., Floss, T., Hansen, J., Altschmied, J., Seisenberger, C., Ghyselinck, N.B., Ruiz, P., Chambon, P., Wurst, W., and von Melchner, H. 2005. *Proc. Natl. Acad. Sci. U.S.A.* 102: 7221–7226.
23. Shen, Q., Sun, Q., Wei, X., Dong, J., Zhang, R., Wu, P., Jin, Y., Feng, J., Li, H., and Hu, Y. 2007. *J. Biosci. Bioeng.* 104: 14–21.
24. Takeuchi, T., Nomura, T., Tsujita, M., Suzuki, M., Fuse, T., Mori, H., and Mishina, M. 2002. *Biochem. Biophys. Res. Commun.* 293: 953–957.
25. Tonegawa, S., Nakazawa, K., and Wilson, M.A. 2003. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 358: 787–795.
26. Yoshiki, A., Ike, F., Mekada, K., Kitaura, Y., Nakata, H., Hiraiwa, N., Mochida, K., Ijuin, M., Kadota, M., Murakami, A., Ogura, A., Abe, K., Moriwaki, K., and Obata, Y. 2009. *Exp. Anim.* 58: 85–96.