Rapid Screening of Transgenic Type II and Type XI Collagen Knock-Out Mice with Three-Primer PCR

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We demonstrate in this article the rapid determination of heterozygous and homozygous transgenic knock-out mice using the polymerase chain reaction (PCR) with three primers. A similar technique was described by Horton et al. (3), which demonstrated three-primer PCR in the genotype determination of knock-out RAG-2 immunodeficient mice. However novel this approach may be, one must conclude that the procedure by Horton is quite time-consuming. This is a rate-limiting step when utilizing transgenic/knock-out mice as a biological tool; most knock-out mice develop fatal phenotypes at birth or within a few hours post-partum. We offer in this article an expeditious approach for screening knock-out mice, for determining the genotype and for achieving satisfactory results within the same day of pup mice birth through the use of three-primer PCR.

The knock-out mice in this study are each deficient for a specific collagen gene. In the collagen II knock-out mouse, there is a deletion of the *COL2A1* gene, which has been inactivated or "knocked out" by insertion of a neomycin-resistance gene. The heterozygous genotype of these mice develops normally, but the homozygous offspring develops a fatal phenotype within minutes after birth (4). In the collagen XI knock-out mouse, there is also a replacement for a portion of the gene with the neomycin-resistance gene in the collagen gene *Col11A2*. The heterozygous phenotype of these mice are normal, but the homozygotes develop limited phenotypes such as bulging eyes and a short snout accompanied by a short body stature. The phenotypes are within themselves an aid in the determination of the genotype, but this does not hold true as in the case of the heterozygous variety of both type II and type XI knock-out mice. To avoid any ambiguity, PCR is the ultimate tool utilized in the determination of the mice genotype. Furthermore, we have found that the use of three primers (Figure 1) in PCR analysis uniquely distinguishes between a heterozygous or homozygous genotype of the mouse of interest. Additionally, this proposed technique serves as an internal control when certain PCRs are prone to yielding nonspecific amplified DNA.

Table 1. Three-Primer PCR with Modified Quick-Screening PCR

1. Toe samples are placed in 1× PCR lysis buffer 10 µL (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 50 mM $MgCl_2$, 0.1% gelatin, 10% Brij 35 [Sigma Chemical, St. Louis, MO, USA]), 4 µL 10 mg/mL proteinase K (Boehringer Mannheim, Indianapolis, IN, USA) and 86 µL distilled H_2O at 55°C for 3 h.
2. Digested samples are then heated in a heat block at 96°C for 10 min and immediately placed on ice.
3. The individual PCR master mixtures consist of 5 µL of 10× PCR buffer (500 mM KCl, 100 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 200 µg/mL gelatin [Sigma Chemical]), 8 µL 1.25 mM dNTP mixture (Pharmacia Biotech, Piscataway, NJ, USA), 1 µL of 15 µM of each primer: E34 (CTGTGCTTATAGGACTCAGG), E36 (GGAGTCAGAG CACTGGTCATG) and Neo-7 (GCTATCAGGA-CATAGCGTTGG) for COL2A1 knock-out mouse, or XI-2 (AAGTGGGAACAC-CGGGAAGT), XI-3 (CTGAGGAGTCTTCAGACTGG) and neo-7 for ColXIA2 knock-out mouse and 0.25 IU of AmpliTaq® DNA polymerase (Perkin-Elmer, Norwalk, CT, USA).
4. Forty-five microliters of this mixture are then aliquoted into 0.2-µL MicroAmp® Reaction Tubes (Perkin-Elmer).
5. Five microliters of toe lysate are then added to each respective tube and capped with MicroAmp Cap (Perkin-Elmer).
6. The samples are then amplified for 30 cycles; each cycle parameter consists of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min in a GeneAmp® PCR System 9600 (Perkin-Elmer).
7. The samples are then separated on a 2.5% agarose gel (1% agarose and 1.5% NuSieve® GTG; FMC BioProducts; Rockland, ME, USA).

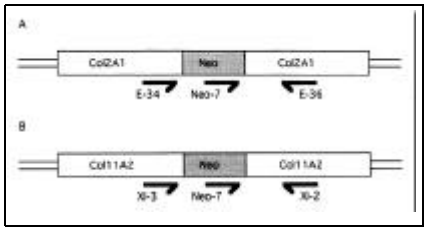

Figure 1. Primers and their position for their respective gene. (a) Mutant *COL2A1* gene and its primer positions (E-36, Neo 7 and E34). (b) Mutant *COL11A2* gene and its primer positions (XI-3, Neo-7 and XI-2).

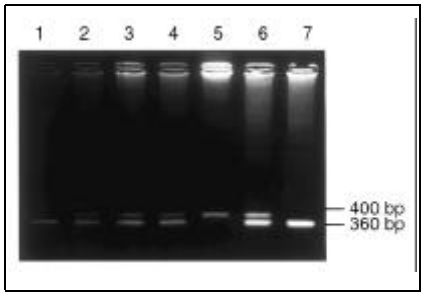

Figure 2. Agarose gel (2.5%) separation of ColXIA2 three-primer PCR product of eight samples. Sample 1, homozygous knock-out COL11A2 (400 bp); samples 2–4, heterozygous (400 and 360 bp); sample 5, normal (360 bp); and samples 6 and 7 are controls, heterozygous and homozygous, respectively.

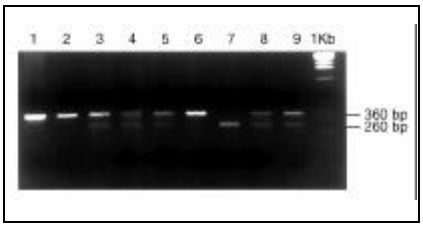

Figure 3. Agarose gel (2.5%) separation of Col2a1 three-primer PCR product of nine samples. Samples 1, 2 and 6, homozygous knock-out COL2A1 (360 bp); samples 3–5, 8 and 9, heterozygous (260 and 360 bp); and sample 7, normal.

According to Arita et al. (1), screening of transgenic mice is expeditiously achieved by toe clipping with subsequent analysis by PCR. This particular procedure allows one not only to achieve favorable results with regards to positive or negative genotypes of the offspring, but also to systematically numerate mouse pups for identification purposes within the same day of birth.

In this experiment, progeny of different and combined matings between heterozygous COL2A1 and heterozygous or homozygous Col11A2 mice were rigidly observed and subjected to toe clipping on the day of birth. The procedure, according to Arita et al. (1), is shown in Table 1.

The transgenic knock-out mice in this study are an invaluable model for the investigation of various types of bone pathology. Early-onset familial osteoarthritis and a heterogeneous group of disorders of cartilage that are known as chondrodysplasias have been linked to over 50 mutations in the *COL2A1* gene (5). Hence, the knock-out collagen type II (COL2A1) mouse is ideal for the study of these specific cartilage pathologies. Non-ocular Stickler syndrome has been found to be linked to the *COL-11A2* gene along with linkage to autosomal dominant and recessive osteochondrodysplasias (2,6). The knock-out collagen Type XI mouse will certainly aid in the investigation of these bone and cartilage disorders. In addition, these mice should prove to be excellent candidates for models in gene therapy study for their respective disorders.

REFERENCES

1. **Arita, M., S.-W. Li, D.C. Bertolette and J.S. Khillan.** 1995. Quick screening of newborn transgenic mouse pups by polymerase chain reaction (PCR). Int. J. Anim. Sci. *10*:1-3.
2. **Brunner, H.G., S.E.C. Van Beersum, M.L. Warman, B.R. Olsen, H.H. Ropers and E.C.M. Mariman.** 1994. A Stickler's syndrome gene is linked to chromosome 6 near the COL11A2 gene. Hum. Mol. Genet. *3*:1561-1564.
3. **Horton, R.M., P.I. Karachunski and B.M. Conti-Fine.** 1995. PCR screening of transgenic RAG-2 "knockout" immunodeficient mice. BioTechniques *19*:690-691.
4. **Li, S.W., D.J. Prockop, H. Helminen, R. Fassler, T. Lapvetelainen, K. Kiraly, A. Peltarri, J. Arokoski, H. Lui, M. Arita and J.S. Khillan.** 1995. Transgenic mice with targeted inactivation of the COL2A1 gene for collagen II develop a skeleton with membraneous and periosteal bone but no endochondrial bone. Genes Dev. *9*:2821-2830.
5. **Ritvaniemi, P., J. Korkko, J. Bonaventure, M. Bikkula, J. Hyland, P. Paassilta, I. Kaitila, H. Kaariainen, et al.** 1995. Identification of COL2A1 gene mutations in patients with chondrodysplasias and familial osteoarthritis. Arthritis Rheum. *38*:999-1004.
6. **Vikkula, M., E.C.M. Mariman, V.C.H. Lui, N.I. Zhidkove, G.E. Tiller, M.B. Goldring, S.E.C. van Beersum, M.C. de Waalmalefijt et al.** 1995. Autosomal dominant and recessive osteochondrodysplasias associated with the COL11A2 locus. Cell *80*:431.

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Improved Screening Method for Recombinant Retrovirus Producing Clones

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Recombinant retroviral vectors for gene therapy purposes have been constructed carrying cDNA copies of genes useful for the treatment of a number of congenital or acquired diseases such as ADA deficiency (6), Gaucher's disease (7) and cancer (suicide genes) (2). In most cases, expression of these cDNAs in a packaging cell (5) does not confer any specific drug resistance phenotype, which hampers the rapid isolation of a high-titer virus producer cell clone. We have developed a screening method, which permits rapid selection and isolation of cell clones producing high amounts of functional recombinant virus carrying a cDNA encoding a non-selectable gene product.

The basic principle of the reported screening method is presented schematically in Figure 1 and has been worked out for retroviral vectors carrying the human lysosomal glucocerebrosidase (hGC) cDNA (4), the enzyme deficient in Gaucher patients. For the detection of the glucocerebrosidase protein, we used a primate-specific monoclonal antibody, 8E4 (1). After infection with an ecotropic recombinant retrovirus, the amphotropic retroviral packaging cells were harvested and plated on 96-well microplates at a theoretical concentration of <1 cell/well (Figure 1, Step 1). To increase efficiency, a 96-well pipetter was used for all handlings involving microplates. After 2–3 weeks, these cells reached 80%–100% confluency, after which the medium was replaced with fresh medium followed by an incubation for 24 h at 32°C to allow optimal virus production (3). Target cells were seeded the day before infection at a density of 2000 cells per well in 96-well microplates. For infection, cell culture supernatant from the producer clones was transferred to the target cells, and fresh medium was added to the producer cell clones (Figure 1, Step 2). The plates containing the producer cell clones were transferred to a CO_2 incubator set at 32°C to prevent the cells from overgrowing. After an infection period of 48 h at 37°C, protein lysates were prepared from the infected target cells. The lysates were transferred to antibody-coated 96-well plates (Figure 1, Step 3). These 96-well plates were prepared the day before by incubation with 10 µg/mL goat anti-mouse antibody (GAM) in phosphate-buffered saline (PBS) for 1 h at 37°C and subsequent coating with 8E4 diluted in PBS/ 0.5% bovine serum albumin (BSA) overnight at 4°C. Between steps, unbound antibodies were removed by washing with PBS. Binding of hGC by 8E4 occurred for 2 h at room temperature after which excess protein was removed by washing with lysis buffer. Glucocerebrosidase activity was detected as described (1).