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Note

**Application of the colorimetric loop-mediated isothermal amplification (LAMP) technique
for genotyping *Cre*-driver mice**

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Running title: LAMP METHOD FOR CRE GENOTYPING

ABSTRACT

The *Cre-loxP* system is widely used to investigate the cell-type specific roles of genes of interest. *Cre*-driver mice are required for cell-type specific knockout during the *Cre-loxP* reaction. To maintain *Cre*-driver mouse strains, Polymerase chain reaction (PCR)-oriented genotyping targeting the *Cre* gene cassette is usually conducted. In this study, we instead applied a colorimetric loop-mediated isothermal amplification (LAMP) method for *Cre*-genotyping. Among four sets of primers designed by the *in silico* program, one set effectively amplified the *Cre* cassette of three *Cre*-driver strains, but not of C57BL/6 mice. This LAMP-oriented method reduces assay time by less than half compared to the PCR-based method, and can be carried out using a conventional isothermal incubator. Applying this LAMP method may accelerate genotyping of *Cre*-driver mice.

KEY WORD

conditional knockout, Cre, genotyping, loop-mediated isothermal amplification

Gene knockout mice are useful tools for investigating pathological and physiological roles of genes of interest at an individual level [2, 10]. To achieve cell type-specific gene knockout, the *Cre-loxP* technique has been widely applied [3, 11]. Various strains of mice having an exogenous *Cre* gene, so-called *Cre*-driver mice, have been generated to assess the roles of particular genes in different types of cells [5]. Repeated sibling mating of *Cre* homozygotes may cause founder effect. In accordance, *Cre*-driver mice bred as homozygote at the *Cre* locus displayed developmental defects or lethality [1, 7]. Therefore, to retain genetic nature of parental strains, *Cre*-driver mice are usually bred as heterozygotes/hemizygotes at the *Cre* locus by mating with the parental strains. With this background, genotyping of the *Cre* locus is routine for preserving the *Cre*-driver mice strains. Polymerase chain reaction (PCR) is the most commonly used technique for genotyping *Cre*-driver mice. To carry out a PCR assay, genomic DNA is mixed with an enzyme solution containing heat-resistant DNA polymerase, a pair of primers, and deoxyribonucleotide triphosphate, and then subjected to cycle reactions using a thermal cycler. After separation, amplification products are visualized using agarose gel electrophoresis. In general, conventional PCR-based genotyping requires two to three hours.

The loop-mediated isothermal amplification (LAMP) method is another valuable method for amplifying nucleotides. LAMP enables amplification of a few copies of DNA to 10^9 copies in less than an hour under a constant temperature [9]. Moreover, amplification by LAMP

reaction is markedly specific since it uses more than four primers for the target DNA. Furthermore, using loop primers increases the LAMP reaction's efficiency and specificity [8]. Because accumulation of amplicon lowers the pH of the reaction mixture, adding pH indicators, such as phenol red, cresol red, and neutral red, enables one to check the degree of amplification and thereby obviates the need for a special device [12, 13]. Despite its potential usability, the LAMP method is not commonly used for genotyping mammalian species (except for sex determination) [4, 6]. Primer design in particular requires further development to facilitate routine use of the LAMP technique. In this study, we attempted to establish a simple and time-effective procedure for genotyping the *Cre* gene in mice using a LAMP-oriented method.

MuCre mice were obtained from the RIKEN Bioresource Center (Tsukuba, Japan). *NesCre* and *FosCre* mice were kindly gifted by Dr. Eiki Takahashi (RIKEN Brain Science Institute, Wako, Japan). Wild type C57BL/6 mice were obtained from SLC (Hamamatsu, Japan). All animal experiments were approved by the Animal Experimental Committee of Rakuno Gakuen University (Approval numbers VH17A7, VH17A8, and VH17A16). Care and management of experimental animals was conducted in compliance with the guideline of animal experiments of Rakuno Gakuen University.

Small pieces (~4 mm²) of mice ear skin were obtained when the mice were ear tagged. Genomic DNA was isolated from the tissues using a KAPA Mouse Genotyping kit at half the

scale of the original instructions. DNA contents in the reaction liquid was determined using a BioSpec-nano (Shimadzu, Kyoto, Japan). One μ l (200-800ng) of the DNA solution was used for LAMP or PCR analyses. The LAMP method was performed using the WarmStart Colorimetric LAMP Master Mix (New England Biolabs, Ipswich, MA, USA) at half the scale of the manual instructions. Briefly, reaction mixtures containing 1.6 μ M forward inner primer (FIP) and backward inner primer (BIP) as well as 0.2 μ M F3 and B3 primers were incubated at 65 °C in a thermal cycler (Model 482, Astec, Fukuoka, Japan) or a hybridization oven (HB-80, Taitec, Koshigaya, Japan) for the indicated period. In some cases, 0.4 μ M of loop primers F and B were also added to the reaction mixture. The LAMP mixtures in the tubes were captured using a digital camera equipped in an iPhone 7 or iPad air 2 (Apple, Cupertino, CA, USA). In some experiments, we conducted conventional PCRs for the *Cre* and *Gapdh* genes in an enzyme mixture including 200 ng of genomic DNA, 5 μ l of 2X GoTaq DNA polymerase (Promega, Madison, MA, USA) and 0.2 μ l of 10 μ M primer mix in a 10 μ l solution. Sequences of the PCR primers were: 5'-GTTGATGCCGGTGAACGTGCAAA-3' and 5'-ATCAGCTACACCAGAGACGGAAA-3' for *Cre* gene, and 5'-GCACAGTCAAGGCCGAGAAT-3' and 5'-GCCTTCTCCATGGTGGTGAA-3' for glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) gene. For amplification of both genes, 35 cycles were performed. One thermal cycle consists of three steps: 95 °C for 30 sec for denaturation, 58 °C for 30 sec for annealing, and 72 °C for 30 sec for extension. For

electrophoretic analysis, 5 μ l of the sample was loaded on 2% agarose gel in a Tris-acetate-EDTA buffer [40 μ M Tris-HCl (pH 8.0), 40 μ M acetate, and 500 mM EDTA] and subsequently stained with 500 ng/ml ethidium bromide at room temperature for 5 min. The bands were visualized using a high performance UV transilluminator (Ultra Violet Products, Cambridge, UK) and were captured by a digital camera equipped in an iPhone 7.

For amplification of the *Cre* gene using the LAMP method, we designed four candidate primer sets corresponding to the deposited sequence of the *Cre* gene (Accession number, X03453) using the Primer Explorer V5 program (<https://primerexplorer.jp>). Sequences of each primer set are listed in Table 1. We first attempted to select primer sets which effectively amplify the target locus. To this end, we used genomic DNA from *FosCre* mice and C57BL/6 mice, in which the *Cre* gene is present and absent, respectively (Fig. 1A). Figure 1B shows the timings of color changes of the LAMP reaction liquid using the designed primer sets. Thirty min after beginning the reaction, all mixtures were still red. At 45 min, the mixture with the *FosCre* sample containing primer set 15 changed from red to yellow while the change was not apparent in the C57BL/6 sample. Similarly, primer set 11 altered the color of the *FosCre* sample, but not the C57BL/6 sample. At 60 min, the color of both the *FosCre* and C57BL/6 samples drastically changed to clear yellow in the presence of primer set 15. However, the yellowing was more marked in the *FosCre* sample compared to the C57BL/6 sample when primer set 11 was used. The mixtures

containing primer sets 11 or 15 turned yellow regardless of genotype after 75 min. Primer sets 1 and 5 failed to change the color of the mixture for at least 75 min, although these primer sets gave nonspecific amplification of the C57BL/6 genome at 90 min. Therefore, primer sets 11 and 15 are likely candidates for genotyping *Cre* even though they yielded non-specific amplification by incubation after 60 min.

Previous reports indicated that loop primers increased efficacy and specificity of the LAMP reaction [8]. To avoid false positive amplification using primer set 11, we added 0.4 μ M of loop primers (Table 1) to the LAMP reaction. As mentioned above, primer set 11 facilitated a change from red to yellow 90 min after the reaction start in the C57BL/6 liquid sample, even though the sample did not have the *Cre* DNA element (Fig. 1C). In stark contrast, the loop primers prevented false positive amplification at least until the 90-min time point. We then classified the primer set 11 combined with the loop primers as primer set 11L. We also tried to amplify the *Cre* cassette using primer set 15 and its loop primers (Table 1). In contrast to primer set 11L, set 15 primers amplified non-specific products despite the inclusion of loop primers (data not shown).

We further validated the reliability of the LAMP method with primer set 11L. To assess this, we used primer set 11L to genotype other *Cre*-driver lines. Similar to *FosCre* mouse-derived DNA, primer set 11L caused a color change from red to yellow when tested on DNA isolated from *NesCre* or *MuCre* mice (Fig. 2A). Genotyping of the *Cre* cassette using the LAMP technique was

confirmed using conventional PCR genotyping, which gave an ~150 bp amplification band during subsequent 2% agarose gel electrophoretic analysis (Fig. 2C). The combination of the PCR and agarose gel-electrophoresis methods required 150 min to produce a result, while the alternative colorimetric LAMP method produced the same result in 60 min.

With the increasing interests in tissue specific functions of each gene, the *Cre*-cassette is monitored in research laboratories in a more generic fashion. By taking advantage of the colorimetric LAMP-oriented technique, we established a procedure for genotyping *Cre*-driver mice. The current LAMP-oriented method shortens the enzymatic reaction time to less than half that of the conventional PCR-oriented method. Moreover, the LAMP-oriented method is cost-effective from a capital investment perspective: instruments required for the colorimetric LAMP method include only an isothermal incubator and a simple digital camera, whereas PCR approaches require a thermal cycler, an electrophoresis chamber, and a gel imaging device. Selecting effective primer sets is the greatest obstacle to using the LAMP reaction for mouse genotyping. Researchers can use our optimized primer set for the *Cre* locus for genotyping *Cre*-driver mice to reduce procedure time and complexity.

In addition to conventional PCR, real time PCR becomes one of the common techniques to detect genetic elements in target cells. As with LAMP method, one real time PCR usually completes ~60 min using several commercially available instruments [14]. Moreover, real time

PCR method is advantageous regarding quantitativity compared with colorimetric LAMP method. Thus, real time PCR method can be applied to evaluate copy number of exogenous gene cassettes in sample genome [14]. On the other hand, real time PCR method has the issues same as conventional PCR method: it requires an expensive instruments which equips a precise thermal cycle system, an optical module for high-sensitive fluorescent detection, and an operating PC. This also causes increase in costs for maintenance or repair of the instruments. Additionally, samples and reagents have to be applied to tubes or plates which are specialize for the instruments. Therefore, there are obvious advantages with cost for assay in LAMP method compared with real time PCR method.

CONFLICT OF INTEREST

The authors have read this manuscript and approved the submission, and declare no conflict of interest.

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FIGURE LEGENDS

Figure 1. Establishment of an effective loop-mediated isothermal amplification (LAMP) primer set for the *Cre* gene cassette

Genomic DNA isolated from *FosCre* (f) or C57BL/6 (b) mice was subjected to either polymerase chain reaction (PCR) (A) or colorimetric LAMP reaction (B-C) to identify the presence of the *Cre* gene. A. An electrophoretic image of the PCR. Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) gene is used as an amplification reference. B. A photographic images of the LAMP reaction using primer sets 1, 5, 11, and 15. C. Photographic images of the LAMP reaction using primer set 11 in the presence (+) or absence (-) of loop primers. Asterisks represent changes in liquid color from red to yellow or orange. Similar results were obtained in three independent experiments.

Figure 2. Validation of primer set 11L using several *Cre*-driver mice strains

A. Colorimetric loop-mediated isothermal amplification (LAMP) genotyping for the *Cre* cassette using primer set 11L was run on genomic DNA of *FosCre* (f), *NesCre* (n), *MuCre* (m), and C57BL/6 mice (b). Photographic images of the tubes of the LAMP product are shown. C. Electrophoretic images of the polymerase chain reaction (PCR) products of the *Cre* or glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) amplification of the same samples used for

226 Fig. 2A. Asterisks indicate changes in liquid color from red to yellow or orange. Similar results
227 were obtained in three independent experiments.

TABLE 1. Sequences of loop-mediated isothermal amplification (LAMP) primers

Set	Primer	Sequence
Set 1	FIP	5'-ATTCAACTTGCACCATGCCGCGCCAGGCGTTTTCTGAG-3'
	BIP	5'-AACCGGAAATGGTTTCCCGCATTTTACTGCCAGACCGCG-3'
	F3	5'-AGAACCTGATGGACAT-3'
	B3	5'-GCATGTTTAGCTGGCCCAA-3
Set 5	FIP	5'ACCGCGCGCCTGAAGATATAGATAACCGGAAATGGTTTCCCG-3
	BIP	5'-TGGGCCAGCTAAACATGCTTCACGCATAACCAGTGAAACAGC-3'
	F3	5'-CGGCATGGTGCAAGTTGAA-3'
	B3	5'-CACGTTACCGGCATCAA-3'
Sets 11&11L	FIP	5'-GTTTTTACTGCCAGACCGCGCCCGCAGAACCTGAAGATGT-3'
	BIP	5'-TGGGCCAGCTAAACATGCTTCACGCATAACCAGTGAAACAGC-3'
	F3	5'-CGGCATGGTGCAAGTTGAA-3'
	B3	5'-CACGTTACCGGCATCAA-3'
Set 11L	Loop F	5'-GTTTTTACTGCCAGACCGCGCCCGCAGAACCTGAAGATGT-3'
	Loop B	5'-GGGCCAGCTAAACATGCTTCACGCATAACCAGTGAAACAGC-3'
Sets 15&15L	FIP	5'-CGGTGCTAACCAGCGTTTTTCGTACGTACTGACGGTGGGA-3'
	BIP	5'-AGAGAAGGCACTTAGCCTGGGGCATCAGCTACACCAGAGACG-3'
	F3	5'-GCCGAAATTGCCAGGATCA-3'
	B3	5'-GACCCGGCAAAACAGGTAG-3'
Set 15L	Loop F	5'-CGGTGCTAACCAGCGTTTTTCGTACGTACTGACGGTGGGA-3'
	Loop B	5'-AGAGAAGGCACTTAGCCTGGGGCATCAGCTACACCAGAGACG-3'

FIP, forward inner primer; BIP, backward inner primer

A **B** **C**

Fig.1 Fujimoto and Kitamura

A **B**

Fig.2 Fujimoto and Kitamura