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Generating Tamoxifen-Inducible Cre Alleles to Investigate Myogenesis in Mice

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Abstract

Gene inactivation has become the gold standard for determining gene function in the mouse. Many genes inactivated in the germ line cause early lethality that precludes phenotypic assessment at a later time point. Conditional gene inactivation using Cre recombinase expressed via a tissue specific promoter/enhancer allows phenotypic analyses of selected tissues, but lacks temporal control. Recent development of the tamoxifen-inducible Cre-ER^{T2} offers both cell type-specific and temporal control of conditional gene inactivation. As an example, we describe the design and step-wise construction of a *Cre-ER^{T2}* knock-in allele at the *Pax7* locus using the recombineering method – *Pax7* is selectively expressed in embryonic muscle progenitors and adult muscle stem cells. The resulting *Pax7-Cre-ER^{T2}* (*Pax7^{CE}*) allele has been successfully applied to embryos and adults for tamoxifen-regulated myogenic lineage tracing and gene inactivation (Nature 460:627–631, 2009; Genesis 48:424–436, 2010).

Keywords

Pax7; Myogenic progenitor; Satellite cell; Lineage tracing; Tamoxifen; Cre; Cre-ER^{T2}; Homologous recombination; Recombineering; Knock-out; Knock-in

1. Introduction

In recent years, Cre-mediated recombination between loxP sites to activate reporter gene expression has been intensely used for lineage tracing in the mouse (1). The advantage of the Cre system is that it also provides versatility for concomitant gene inactivation of conditional alleles with loxP sites. However, conventional Cre is constitutively active and thus, its application to reporter gene activation and/or conditional gene inactivation is a cumulative assay for all cells at any one time expressing Cre.

The advanced Cre technology utilizes a tamoxifen inducible Cre-estrogen receptor (ER) fusion protein (2, 3). For this, Cre is fused with the ligand-binding domain of a mutated ligand-binding domain of ER, which does not bind to estrogen but binds to tamoxifen with high affinity. Cre-ER proteins are sequestered in the cytoplasm via association with the HSP90 chaperone. Upon addition of tamoxifen, tamoxifen-bound Cre-ER dissociates from HSP90, translocates into the nucleus, and carries out site-specific recombination between flanking loxP sites. A more advanced version is Cre-ER^{T2}, in which additional mutations have been engineered into the ER ligand-binding domain to bind tamoxifen with a higher affinity (4). Therefore, lower doses of tamoxifen can be used, reducing unwanted side effects – tamoxifen is also an inhibitor to protein kinase C (5).

For manipulating adult muscle stem cells, two *Cre-ER* knock-in (KI) alleles at the *Pax7* locus have been reported (6–8). Below we detail the construction of the KI/knock-out (KO) allele of the *Pax7-Cre-ER^{T2}* allele (6, 7). We contrast our design to an earlier version of the

Pax7-IRES-Cre-ER^{T2} allele (8) in both the main text and the note section. All steps described here are generally applicable to place *Cre-ER^{T2}* into any gene of interest.

2. Materials

There are two major steps in making a *Pax7-Cre-ER^{T2}* homologous recombination vector: (1) Sub-clone a genomic fragment of *Pax7* from a bacterial artificial chromosome (BAC); (2) Place the *Cre-ER^{T2}* and a companion positive selection cassette into the desired position (see later) of the vector containing the genomic fragment of *Pax7*.

1. BAC with ample sequences flanking the location (~10–20 kb on each side) where you plan to place *Cre-ER^{T2}* into your favorite gene. We screened a 129sv genomic library for *Pax7*-containing BACs (Invitrogen). Now the Ensembl genome browser can be searched for 129sv BACs made from AB2.2 ES cells to obtain the target *Pax7* genomic fragment. This BAC library has been end-sequenced and is publicly available (9). Instructions for searching the database and ordering end-sequenced BACs are detailed in the referenced paper (see Note 1).
2. Recombineering reagents: We recommend you familiarize yourself with the recombineering method (10, 11) for making homologous recombination or transgenic vectors. The protocols and reagents are described in the above two references. Login to the following website: <http://web.ncifcrf.gov/research/brb/reagents/recombineeringReagent.aspx> to request reagents and download protocols. Recombineering is ideally suited for this type of cloning as it does not depend on restriction enzyme sites to place *Cre-ER^{T2}* into a desired location (see Note 2). The basic components needed are as follows:
 1. W102 bacteria strain, which contains heat-shock inducible λ phage genes *exo*, *bet*, and *gam* for recombination (see Note 3).
 2. pL253 plasmid, which contains a *tk* cassette for negative selection in ES cells.
 3. A *Cre-ER^{T2}-IRES-DsRed-Frt-neo-Frt* (6) or a *Cre-ER^{T2}-Frt-neo-Frt* cassette. Both cassettes are available from our lab. You may find/order more advanced versions from Addgene (see Note 4).
 4. Electroporator.
 5. Gene-specific oligonucleotides for recombineering (see Method for their design).
 6. T4 polynucleotide kinase.
 7. Agarose.
 8. Ethidium bromide, stock at 10 mg/mL.
 9. Qiagen large construct kit.
 10. Zymogen gel recovery kit.
 11. Luria Broth (LB): 10 g tryptone, 5 g yeast extract, 10 g NaCl per liter of H₂O.
 12. LB-chloramphenicol (LB-chlor, 12.5 μ g/mL chloramphenicol) plates.
 13. LB-ampicillin (LB-amp, 50 μ g/mL ampicillin) plates.
 14. LB-kanamycin (LB-kan, 25 μ g/mL kanamycin) plates.
 15. 32°C incubator.
 16. 32°C shaker.

17. 37°C incubator.
18. 42°C water bath.

3. Methods

3.1. Confirm the Ordered BAC

Based on end sequence information, an in silico prediction of the restriction enzyme map of the BAC is necessary for two reasons: It helps to confirm the BAC to be correct when it arrives; it helps to design restriction enzyme digests and probes for Southern analysis of potential ES cell clones.

1. BAC clones arrive as bacterial stabs. Streak out the bacteria on a LB-chlor plate. The BAC library referenced is constructed in the BACe3.6 vector (chloramphenicol resistant).
2. Inoculate five colonies for mini-prep using standard alkaline lysis method (12). Digest ½ of the miniprep DNA with a restriction enzyme chosen based on the in silico predicted map and run the digested DNA on a 1% agarose gel.
3. Stain the gel with ethidium bromide (5 µg/mL) and check digested patterns for two things: First, all or most of the five clones give you the same pattern (i.e., the BAC is not a mixture); Second, the digest pattern is as predicted based on the in silico analysis (i.e., no gross rearrangements).
4. Inoculate a clone with the predicted digest pattern for a 500 mL prep. The Qiagen large-construct kit can be used. Intact clean BAC DNA is needed for electroporation into SW102 bacteria for recombineering. To be certain that the BAC is as ordered, resequence BAC ends with T7 and Sp6 primers.

3.2. Transfer the BAC into SW102

1. Inoculate a single colony of SW102 in 5 mL of LB in a 14 mL snap cap tube. Grow at 32°C until OD₆₀₀ = 0.4–0.6. Chill on ice for 5 min. Keep cold during the following steps.
2. Spin down the bacteria in a prechilled centrifuge (4°C, 4,000 rpm (Sorvall RC-3B centrifuge), 5 min), and wash with 5 mL of ice-cold deionized water. Repeat the spin-wash step 3 times. After the third wash, resuspend bacteria in 50 µL of deionized water.
3. Electroporate 100 ng of the BAC (from Subheading 3.1) into SW102. Any electroporator designed for bacteria transformation should work for this step. Dilute the electroporated bacteria into 1 mL of LB, incubate at 32°C for 1 h in a shaker.
4. Plate 100 µL onto one LB-chlor plate, and the remainder onto an additional plate. Incubate at 32°C for 24 h.
5. Pick three colonies for mini-prep and perform restriction enzyme digest as above in Subheading 3.1 to be sure that the transferred BAC remains intact.
6. Make a glycerol stock of the BAC clone in SW102 for long-term storage, or proceed to the next step.

3.3. Prepare Oligos and pL253 Vector to Capture Desired Genomic Region from the BAC

The *Pax7-Cre-ER^{T2}* allele that we generated is a KI allele as well as a KO allele. We replaced exon 1 of *Pax7* with *Cre-ER^{T2}* (see Fig. 1). For this, we retrieved a genomic fragment containing ~18 kb 5' arm and ~2 kb 3' arm (see Note 5). If you do not wish to

inactivate the gene function, place the *Cre-ER^{T2}* cassette in the 3' UTR for bicistronic expression via IRES, e.g., the *Pax7-IRES-Cre-ER^T* allele (8) (see Note 6).

pL253 contains a polylinker for linearizing the final vector prior to transfection into ES cells. It also contains a tk negative selection cassette to reduce the number of ES cell colonies containing random insertions.

1. Make (order) four oligonucleotides (see Fig. 1 for configuration): The two 5' oligos are complementary but with a NotI overhang for ligation to NotI-cut pL253. The two 3' oligos are to be ligated to BamHI-cut pL253. For both, we designed 65 bp oligos identical to the 5' and 3' ends of *Pax7* genomic fragment to be retrieved from the BAC (see Note 7).
2. Resuspend each oligo at 1 µg/µL in water. For each pair, set up kinase reaction (using T4 polynucleotide kinase) with 5 µg of the oligo with the restriction enzyme site overhang using standard conditions (12) in a 45 µL reaction.
3. After the kinase reaction, add 5 µg of the corresponding complementary oligo. Immediately boil them together for 5 min. Let them gradually cool to room temp to anneal. Now, they are ready to use at 200 ng/µL.
4. Cut 3 µg of pL253 with NotI and BamHI. Run a 1% agarose gel and gel purify the linearized vector using the Zymogen gel recovery kit and elute DNA in 10 µL of water. Save 1/10 of this for step 6 below.
5. Ligate 9 µL of the linearized vector with 5 µL (1 µg) each of the 5' and 3' annealed oligonucleotides in a 50 µL reaction, overnight at 16°C.
6. Run out the ligation mix on a 1% agarose gel. Run the saved unligated linearized vector (from step 3) side by side as control. There are many bands when the ligation products are run out on the gel, including the ligated vector multi-mers and oligo dimers.
7. Gel isolate the ligated vector, which is only slightly larger than the unligated vector control, using the Zymogen kit and elute DNA in 6 µL of water.
8. Determine the DNA concentration. Minimally 100 ng/µL is needed. The ligated vector is now ready for electroporation into SW102 containing the BAC (from Subheading 3.2).

3.4. Capture the Genomic Fragment from BAC by Recombineering

1. Grow up 5 mL of SW102 bacteria with the *Pax7* containing BAC in LB-chlor (12.5 µg/mL) at 32°C until OD₆₀₀ = 0.4–0.6.
2. Heat shock in 42°C water bath (to induce λ phage recombination system) for 15 min with occasional swirling.
3. Immediately chill on ice and proceed to make competent cells as in Subheading 3.2, Step 2.
4. Electroporate 1–4 µL of purified oligo-ligated vector (preferably 400 ng) into competent cells.
5. Transfer electroporated cells into 1 mL of LB and culture at 32°C for 1 h.
6. Plate the bacteria onto two LB-amp plates, one with 100 µL, and the other with the rest. Incubate at 32°C for at least 24 h.

3.5. Screen for Recombinants

1. Inoculate 5–10 of the smallest colonies in LB-amp and grow them at 32°C until saturation. Bacteria carrying recombined plasmids grow very slowly compared with those carrying recircularized pL253.
2. Isolate DNA by standard alkaline lysis miniprep (12).
3. Without enzyme digest, run 1/5 of miniprep on a 1% agarose gel with size markers. The potential recombinants have only one band of large size (> 10 kb). Those that have a prominent 4–5 kb band are clones with recircularized pL253.
4. Analyze potential recombinants by restriction enzyme digest to determine whether they have the predicted digest pattern. To be sure, make primers flanking the polylinker of pL253 for sequencing the recombined junction.
5. Make a glycerol stock of the SW102 bacteria harboring the recombined plasmid. The bacteria can be used directly for the following step.

3.6. Replace Exon 1 of Pax7 with a Cre-ER^{T2}-Frt-neo-Frt Cassette

The *Cre-ER^{T2}* cassette developed by the Chambon lab (4) has a rabbit globin intron and a SV40 early polyA signal. Both are important for efficient nuclear export of the transcript. We placed an IRES-DsRed between *Cre-ER^{T2}* and the polyA signal for visualizing *Pax7*-expressing cells. For ES cell selection, we included an *Frt-neo-Frt* cassette (derived from the pL452 plasmid (10)) 3' to the polyA signal to make it as one cassette (6). The final cassette has 5' SpeI and 3' SalI sites for oligo ligation/recombineering (see Note 8).

1. Similar to capturing the genomic fragment (same principle as in Subheading 3.3), make four oligonucleotides. The 5' pair of oligos is complementary with a SpeI overhang. The 3' pair of oligos has a SalI overhang (see Note 8).
2. Resuspend, kinase, and anneal the oligos as in Subheading 3.3.
3. Digest 3 µg of *Cre-ER^{T2}-IRES-DsRed-Frt-neo-Frt* (6) cassette with SpeI and SalI, and isolate the digested cassette using the Zymogen kit.
4. Ligate oligos to the cassette.
5. Gel purify the ligated cassette using the Zymogen kit, and elute in 6 µL of water.
6. Prepare electrocompetent cells from heat-shocked SW102 containing the captured *Pax7* genomic fragment in pL253 (from Subheading 3.5) using the same method described in Subheading 3.4.
7. Electroporate the ligated cassette into competent SW102, add 1 mL of LB and incubate for 1 h at 32°C.
8. Plate at two dilutions, 100 and 900 µL, onto LB-kan plates (the neo gene provides kan resistance). Incubate for at least 24 h at 32°C.

3.7. Screen for Recombinants

1. Pick 5–10 of the smallest colonies for culture in LB-kan at 32°C.
2. Miniprep and run 1/5 of DNA on a 1% agarose gel without enzyme digest.
3. For those with only one large band (potential recombinants), dilute 1 µL of DNA in 100 µL of water.

4. Use 1 μ L of each diluted DNA of potential recombinants (3–5 are usually sufficient) for transformation into regular chemically competent cells (e.g., TOP10 or DH5 α). Plate on LB-kan plates, and incubate overnight at 37°C.
5. Pick one colony from each transformation for culture and miniprep. Digest the DNA with the selected restriction enzyme(s) to check for correct recombination. Unlike the BAC, pL253 is a multi-copy plasmid and presumably only one of the multiple plasmids goes through recombination. Thus, the above recombineering step results in mixed plasmids in the bacteria. Retransformation is necessary to remove unrecombined plasmids (not kan-resistant) before analysis by restriction enzyme digest pattern can be carried out accurately.

3.8. Sequence the Recombined Construct

To be sure the construct is the correct recombinant before electroporating it into ES cells, sequence the targeting vector.

1. Sequence the junctions between pL253 and the genomic fragment.
2. Sequence the junctions between *Cre-ERT²-IRES-DsRed-Frt-neo-Frt* and its flanking regions.

If all junction sequences and restriction enzyme digest patterns are as predicted, then the final vector is done.

3.9. Additional Steps

To obtain the mouse line, you need to carry out additional steps. As these steps are routine now, we only point out some main points needing attention. Your transgenic facility manager should also be able to help you out with the steps below.

1. Make primers to PCR across the short arm to screen for homologous recombination events in ES cell clones: one inside in the *Cre-ERT²-IRES-DsRed-Frt-neo-Frt* cassette, and the other outside of the homology arm.
2. Design Southern probes located outside of homology arms to determine true homologous recombination. PCR across the short arm is only for quick screening. Southern analysis should be done for confirmation, preferably on both ends.
3. Remove the *Frt-neo-Frt* cassette either by transfecting a *FLPe* expressing plasmid (available from Addgene) into the targeted ES cells (and select for clones that have the neo cassette deleted) prior to blastocyst injection or by crossing to the Actin-*FLPe* mice (available from Jackson Lab) after germ line transmission of the allele.
4. Cross the *Cre-ERT²* allele to a Cre reporter line, e.g., *Rosa26RLacZ* or *Rosa26R γ FP* (available from Jackson Lab) to test its efficacy (see Note 9).
5. Preparing tamoxifen: Tamoxifen can be made in corn oil at 20 mg/mL, aliquoted, and stored in –80°C. Thawed tamoxifen can be stored at 4°C and used up to 2 weeks with full activity (see Note 10).
6. Administering tamoxifen: The dosage of tamoxifen needs to be titrated pending on your experimental needs. For the *Pax7-Cre-ERT²* allele, we used 1.5–3 mg/40 g of mouse body weight. Intraperitoneal injection of tamoxifen is the easiest and requires only minimal training. Oral gavages are less harmful to the animal but require a bit more training. Food supplementation is also an option – contact Harland Tek for more information on manufacturing customized food.

4. Notes

1. Isogenic DNA is preferred for homologous recombination in ES cells. If your transgenic facility uses a 129sv derived ES cell line, buy the BAC from the AB2.2 BAC library. C57/BL6 ES cells are becoming more widely used. There are several C57/BL6 BAC libraries listed on the CHORI (Children's Hospital Oakland Research Institute) website. You can use the UC Santa Cruz Genome Browser website to search for CHORI BACs containing your genes of interest. The website has a direct link to paired-end sequenced BAC clones for purchase.
2. There are newer versions of recombineering methods and materials from other labs. They have become more sophisticated and easier to use. For example, the method developed by the Capecchi lab (13) may be easier to use for certain kinds of targeting vectors.
3. When we did recombineering for the *Pax7-Cre-ER^{T2}* vector, NCI provided the DY380 bacteria (discontinued) for recombineering. It has been substituted by SW102, hence we use SW102 in the text. There are also other updated changes incorporated into this protocol, which are not always explicitly stated.
4. Addgene has many published vectors deposited for public distribution. We find it easier to get plasmids from Addgene than to ask from investigators.
5. The long and short of the homology arms: We now design all recombination constructs with a very long arm and a less than 2 kb short arm. Arms less than 2 kb are consistently more reliable for PCR screening of the homologous recombinant. If your lab is proficient in using Southern hybridization for screening ES cells, then you can make both arms as long as you wish (note that high copy number plasmids have an upper size limit around 27–30 kb for propagation). We cannot emphasize enough that a Southern is essential to be certain of correct homologous recombination. Because the particular place you “have to” place the *Cre-ER^{T2}* cassette, you may not have many choices for restriction enzymes. Hence, a careful in silico investigation prior to starting a project is crucial.
6. To KO or not to KO when creating a KI: You can insert the *Cre-ER^{T2}* cassette after the stop codon and employ the IRES to express *Cre-ER^{T2}*, which is followed by the gene's own 3' UTR. A *Frt-neo-Frt* cassette can be placed behind the *Cre-ER^{T2}* cassette for positive selection in ES cells. The advantages of this approach are that the function of the gene is retained and that the endogenous 3' UTR is utilized. As microRNA regulation is prevalent, preservation of the natural 3' UTR offers an additional layer of control for regulated expression. On the flip side, the use of IRES often leads to a lower level of expression of the downstream gene (the *Cre-ER^{T2}*). For this to work as intended, you should not put an artificial polyA signal after *IRES-Cre-ER^{T2}* and you should remove the *Frt-neo-Frt* cassette.

The *Pax7-Cre-ER^{T2}* allele described here is also a KO allele (6, 7): The advantage is that the *Cre-ER^{T2}* is likely expressed at a higher level than that of utilizing IRES. The disadvantages are that the mouse is heterozygous for *Pax7* and that the 3' UTR is artificial.
7. The design principle of the oligonucleotides: Although 45 bp of homology is said to be sufficient for recombineering, we find that in practice 65 bp in length is better. If you experience difficulty to obtain recombinants using oligonucleotides, then use PCR to generate larger homology fragments. We had to do so for the second step. 300–400 bp fragments for both sides can increase efficiency dramatically. Incorporate the restriction sites at the same end as the

oligonucleotides. In this case, cut the PCR fragment with restriction enzymes so that they can be ligated to the cassette. We recommend you draw out each recombination step with your designed oligonucleotides and target sequences to make sure everything is in the right order. If you place the overhang at the wrong end of the oligonucleotides, you will not get the recombination events that you plan.

8. In our *Pax7-Cre-ER^{T2}* mice, Ds-Red was not detected, likely due to inefficiency from IRES-mediated bicistronic expression. Thus, “over-design” does not always pay off.

There are other cassettes deposited at Addgene and you should check for updated versions that suit your need. We recently made a simpler cassette with two polyA signal sequences (one SV40 late polyA followed by SV40 early polyA), which hypothetically should increase the efficiency of polyA addition. This new cassette has 5′ NotI and 3′ SalI sites for ligation/recombineering. Both cassettes are diagrammed in Fig. 1 and are available upon request.

9. The keys to characterize a new *Cre-ER^{T2}* line are the following:
 - a. Set up crosses to obtain mice carrying both your *Cre-ER^{T2}* and a reporter allele. Without administering tamoxifen, there should be no reporter gene expression, i.e., no leaky Cre activity. For example, the *Pax7-IRES-Cre-ER^T* mouse is reported to have spontaneous activity, though minimal (8).
 - b. Choose the Rosa26 reporter allele: there are many alleles with different reporters available from the Jackson Lab. The original *Rosa26RLacZ* allele is still popular because of the simplicity of detecting reporter gene expression by X-gal staining. For those who are interested in cellular morphology or live imaging, fluorescent reporter alleles may be favored.
10. You should titrate the dosage of tamoxifen for your experiment. Because each *Cre-ER^{T2}* line expresses different levels of Cre in different cell types, different tamoxifen dosages will likely be required to achieve your goal. For example, a single low dosage of tamoxifen may be preferred for clonal lineage tracing. On the contrary, multiple injections are likely needed to efficiently inactivate conditional alleles.

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Fig. 1.

Flow chart of creating a *Cre-ER^{T2}* knock-in targeting vector. The two recombineering steps are outlined. Vectors, cassettes, and pertinent elements of the constructs are as depicted and labeled; oligos are *short lines*; *squares*, restriction enzyme site overhangs; p, phosphorylated ends. We recommend you draw out the recombination events so that the oligonucleotide sequences/orientations and the overhangs are placed in the correct order for ligation and recombineering. At the *bottom*, a simpler *Cre-ER^{T2}* cassette is diagrammed (see Note 8) and can be used as an alternative.