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***Basic Study***

**Construct of Gpm6a/ReelinGFPCreERT2 by BAC recombineering using specific gene in hepatic mesothelial or stellate cells**

Shi HB *et al.* Gpm6a/ReelinGFPCreERT2 construct

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**Author contributions:** Shi HB and Lou JL contribute equally to this work; Shi HB and Shi HL carried out most of the experiments; Ren F purified the strain and performed PCR; Chen Y analyzed the pattern of restriction enzyme digestion; Shi HB drafted the manuscript and Lou JL analyzed the experimental data; Duan ZP and Lou JL conceived and supervised the study; Duan ZP was involved in editing the manuscript; all authors read and approved the final manuscript.

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**Abstract**

***AIM***

To prepare Gpm6a/ReelinGFPCreERT2 construct by a rapid and reliable strategy using bacterial artificial chromosome (BAC).

***METHODS***

Gpm6a and Reelin BAC were purified and transformed into SW102 *E.coli* by electroporation. GFPCreERT2 fragment was prepared from shuttle vector and transformed into SW102 *E.coli* carrying BAC. Homologous recombination was induced in SW102 *E.coli.* Recombinant clones were screened and confirmed by PCR and restriction enzyme digestion. Recombinant clones were transformed into SW102 *E.coli* to remove Kanamycin unit*.*

***RESULTS***

Complete BAC was transformed into SW102 *E.coli* by electroporation successfully, because BAC purified from SW102 *E.coli* showed the same pattern as original BAC with BamHI digestion. GFPCreERT2 fragment was prepared successfully because we obtained the same size fragment as expected. Homologous recombination was induced and GFPCreERT2 was inserted into the correct site of BAC, because we found the band change was same as the expected pattern after restriction enzyme digestion. Kanamycin unit was removed because we got different sizes of bands consistent with expected by PCR with different primers.

***CONCLUSION***

The construct of Gpm6a GFPCreERT2 or ReelinGFPCreERT2 was prepared successfully, which will establish foundation for tracing the hepatic stellate cells lineage and studying its function.

**Key words:** Bacterial artificial chromosome; Homologous recombination; Glycoprotein M6a; Reelin

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**Core tip:** Up to now, there are few specific mouse lines which can cause recombination for tracing hepatic mesothelial cells or hepatic stellate cells. Here, we describe a rapid and reliable strategy for construct preparation using bacterial artificial chromosome. This study prepared for the first time Gpm6a/ReelinGFPCreERT2 construct, which is the first step for the preparation of Gpm6a GFPCreERT2 or ReelinGFPCreERT2 mouse line.

Shi HB, Lou JL, Shi HL, Ren F, Chen Y, Duan ZP. Preparation and identification of Gpm6a/ReelinGFPCreERT2 construct. *World J Gastroenterol* 2016; In press

**INTRODUCTION**

Excessive extracellular matrix (ECM) of the liver results in cirrhosis, the end stage liver disease of high mortality for which efficacious medical treatments are not currently available except for liver transplantation. Hepatic stellate cells (HSCs) activation is considered a major mechanism in the formation of fibrosis and cirrhosis. However fundamental questions concerning cell fate regulation of HSCs remain largely underexplored. Recent study reported that hepatic mesothelial cells are the potential precursors for HSCs in the development of liver and can transdifferentiate into myofibroblast cells in mouse liver fibrosis [1-3].

Up to now, there are few specific mouse lines which can cause recombination (Cre) for tracing hepatic mesothelial cells or hepatic stellate cells (HSCs). The Wt1CreERT2 mice are useful to trace hepatic mesothelial cells, but the labeling efficiency and specificity is low [4-6]. Specific genes have been identified in hepatic mesothelial cells and HSCs by microarray [7,8]. We will make Cre mouse lines with the specific markers for the study of HSCs or hepatic mesothelial cells. Based on the previous study, glycoprotein M6a (Gpm6a) has been identified as a specific surface marker of hepatic mesothelial cell. It covers on the surface of liver and migrates from surface into the center [4]. Reelin is extracellular matrix glycoprotein, which is a specific HSCs marker in mouse liver and has similar amounts in resting and activated HSCs [9].

Gpm6a GFPCreERT2 or ReelinGFPCreERT2 mouse line will express a fusion protein of green fluorescent protein (GFP), Cre recombinase (Cre), and estrogen receptor induced by tamoxifen (ERT2) under the control of the Gpm6a or Reelin promoter. GFP is used to trace the labeled protein as a marker. Cre recombinase is used to delete a segment of DNA flanked by LoxP sites (flox). ERT2 system is used to activate Cre activity by tamoxifen treatment [10,11].

Cre mouse lines are very useful tools, which can generate knockout mice through the crossover between Cre and flox mouse lines. We need a mouse line to trace the hepatic stellate cells lineage and study its function through knockout of specific gene in specific cells, so it is very necessary to prepare the Gpm6a GFPCreERT2 or ReelinGFPCreERT2 mouse line.In this study, we will investigate the preparation and identification of Gpm6a/ReelinGFPCreERT2 construct, which is the first step for the preparation of Gpm6a GFPCreERT2 or ReelinGFPCreERT2 mouse line.

**MATERIALS AND METHODS**

***BAC DNA clones and shuttle vector***

Gpm6a BAC (RP23-410D17) and Reelin BAC (RP23-143M9) were purchased from BACPAC Resource Center (BPRC) located at the [Children's Hospital Oakland Research Institute](http://www.chori.org/) in Oakland, California, United States. Female (C57BL/6J) mouse BAC library is made from kidney and brain DNA, cloned into the pBACe3.6 vector in the EcoRI site and transformed into DH10B *E.coli*.The reporter gene GFPCreERT2 is in the shuttle vector which was developed by Biomed Company. 5arm and 3arm were inserted into both sites of the reporter gene. The arm sequences were amplified according to the BAC template by polymerase chain reaction (PCR).

***Purification of BAC DNA***

Streak DH10B *E.coli* onto LB plates with 25 mg/mL Chloramphenicol (Cmr, Sigma, United States) at 32 oC. Pick up single colony and culture in 25 mL LB medium with Cmr. Purify BAC DNA with large construct kit (QIAGEN, Germany). Confirm BAC DNA with restriction enzymes BamHI (Biolabs, New England).

***Electroporation of BAC DNA into SW102 E. coli***

Streak SW102 *E.coli* (NCI at Frederick, United States) onto plates with 50 mg/mL Tetracycline (Tc, Sigma, United States) at 32 oC. Pick up single colony and inoculate in LB medium with Tc for 4-6 h. Place on ice and make competent cells from SW102 *E. coli*. Wash with ice-cold water and Add 5 mL BAC DNA into 85 mL competent cells. Electroporate by Gene Pulser Xcell (Biorad, United States) at 1.75 KV, 25 mF (time constant: 4.5-5.0). Add 1 m LB and incubate at 32 oC for 1 h. Plate onto LB plates with Cmr and purify BAC using QIAGEN kit. Confirm BAC DNA with restriction enzymes BamHI.

***Preparation of reporter gene GFPCreERT2***

Streak SW102 *E.coli* onto 50 mg/mL kanamycin (Kam, Sigma, USA) plates at 32 oC. Pick up single colony and inoculate in LB medium with Kam for 4-6 h. Purify the shuttle vector with Endofree plasmid maxi kit (QIAGEN, Germany). Digest the vector with NotI and FseI (Biolabs, New England) and make electrophoresis with agarose gel (Takara, Japan). Recover the big fragment of GFPCreERT2 from the gel according to quick gel extraction kit (QIAGEN, Germany).

***Homologous DNA recombination***

Streak SW102 *E.coli* carrying BAC onto LB plates with 50 mg/mL Tc and 25 mg/mL Cmr. Induce SW102 *E.coli* carrying BAC and make competent cells as mentioned above. Add 200 ng of fragment of GFPCreERT2 into 85 mL competent cells. Electroporate at 1.75 KV, 25 mF (time constant: 4.5-5.0). Add 0.6 ml LB and culture at 32 oC for 1 h. Plate onto LB plates with 25 mg/ml Cmr and 12.5 mg/mL Kam. Confirm recombinant BAC DNA by PCR with platinum Taq DNA Polymerase (Invitrogen, United States) and restriction enzymes digestion.

***Removal of Kam unit and selection of deleted Kam clone***

Streak SW105 *E.coli* (NCI at Frederick, United States) without antibiotics. Pick up single colony and make competent cells. Transform recombinant BAC DNA into competent cells with electroporation. Inoculate in LB medium with L-arabinose (Sigma, United States) for removal of Kam unit. Plate onto LB plates with Cmr only. Check no colonies in LB plate with Cmr and Kam. Pick up 16 colonies and check by PCR using 5 arm primer. Confirm one colony by PCR using different primers. Digest the PCR product with BamHI and AflII (Biolabs, New England) for further confirmation.

**RESULTS**

***Conformation of BAC with restriction enzyme BamHI******digestion***

As we mentioned before, we purchased BAC from BPRC. We confirmed the BAC sequence at the first. From Figure 1, we can see that after digestion with BamHI, Gpm6a BAC had 11 bands, which was same as expected pattern. For Reelin BAC, the same results were got. So we thought the BAC clone we purchased was what we want.

***Confirmation of BAC DNA transformed into SW102 E.coli***

BAC DNA is so long that it is impossible to transform BAC into SW102 *E.coli* bychemical transduction [12]. We transformed purified BAC into SW102 *E.coli* byelectroporation. After electroporation, we purified BAC from SW102 *E.coli* and indentified BAC with restriction enzyme BamHI digestion. For Gpm6a BAC, after BamHI digestion, selected clones did not show the same digestion pattern with original BAC as Figure 2, which suggested original BAC DNA may be fragmented during purification step. For Reelin BAC, we got two positive clones which showed the same pattern as original BAC after BamHI digestion. We improved the purification methods and finally we succeeded in transforming complete Gpm6a BAC to SW102 *E.coli* (Figure 2B).

***Preparation of reporter gene GFPCreERT2***

The reporter gene GFPCreERT2 is in the shuttle vector which contains some genes encoding 5arm, *flp* recombinase target (FRT), kanamycin, FRT, GFPCreERT2, 3arm and ampicillin. We used NotI and FseI to digest the shuttle vector and got the fragment of GFPCreERT2 flanked by 5arm and 3arm. As shown in Figure 3, after digestion with NotI and FseI, we got two bands in which one was about 7600bp and the other was about 2800bp. We recovered the long band containing GFPCreERT2 from agarose gel for transformation.

***Screening and confirmation of recombinant clones***

We induced the SW102 *E.coli*carrying BAC to be competent cells and transformed the fragment of GFPCreERT2 into the SW102 *E.coli* carrying BAC. After homologous recombination between BAC and GFPCreERT2, GFPCreERT2 replaced the first extron in BAC vector. Then we purified the recombinant BAC for screening and confirmation. As shown in Figure 4B, we got 14 positive clones for Gpm6a (14/25, 56%) by PCR screening. For Reelin, we got 22 positive clones (22/25, 88%). We further confirmed the positive clones by restriction enzyme digestion. For Gpm6a, we found the band change was same as the expected pattern after BamHI or KpnI digestion (Figure 4C). Reelin had the same results (Figure 4D). It suggested that GFPCreERT2 was inserted into the correct site in BAC and we got the recombinant BAC.

***Screening and confirmation of recombinant clones without kanamycin gene***

Finally, we must remove the kanamycin unit which may interfere with the expression of reporter gene. We purified the recombinant BAC carrying GFPCreERT2 and transformed it to SW105 *E. coli*. Kanamycin unit was removed by homologous recombination. As shown in Figure 5B, we got 16 positive clones for Gpm6a (16/16, 100%) by PCR screening. We further confirmed the positive clones by PCR with different primers. We got different sizes of bands which were consistent with expected (Figure 5C). We digested the PCR product of 4527bp with BamHI or AflII, and the bands we got were the same as expected (Figure 5D). It suggested that kanamycin unit was removed and we got the deleted kanamycin BAC carrying GFPCreERT2.

***Conclusion***

First of all, we need to choose a vector to prepare the construct of Gpm6a/ReelinGFPCreERT2. For conventional vector, its advantage is easy to handle, but it has low expression of the reporter genes and low specificity because DNA size is about 3-5kb containing only promoter. For bacterial artificial chromosome (BAC) vector, its advantages include relatively high efficiency and relatively high specificity, but BAC DNA is fragile and can break easily because BAC DNA is about 200kb containing all regulatory sequences upstream and downstream of the encoding sequence[13, 14]. Thus we choose BAC vector to prepare the construct of Gpm6a GFPCreERT2 and ReelinGFPCreERT2.

Secondly, how to insert reporter genes into BAC DNA? In 200 kb BAC DNA, there would be 49 recognition sites for 6 nucleotide cutters such as EcoRI and BamHI, so it is impossible to use restriction enzymes digestion and ligation. We use homologous DNA recombination for gene insertion [15-18]. In BAC DNA, we design the primers in upstream or downstream of the first extron and get 5arm and 3arm fragment by PCR amplification. In the shuttle vector, 5arm fragment is inserted into the upstream of the reporter gene and 3arm fragment is inserted the downstream of the reporter gene. In SW102 *E. coli*, the reporter gene that is GFPCreERT2 will replace the first extron in BAC vector by homologous recombination. The expression of GFPCreERT2 is regulated by Gpm6a or Reelin promoter.

Finally, we removed kanamycin unit by homologous recombination of *flp* recombinase target (FRT) using SW105 *E. coli*. The FRT is similar to LoxP which is used to delete a segment of DNA flanked by LoxP sites [19]. The FRT cassette is excised with high frequency which is close to 100% [20]. Then we got the construct of Gpm6a GFPCreERT2 and ReelinGFPCreERT2 successfully. After microinjection, we can get the mouse line with Gpm6a GFPCreERT2 and ReelinGFPCreERT2.

Homologous recombination was performed by modified *E.coli* bacteria strain: SW102 and SW105. SW102 *E.coli* carries some genes such exo, bet and gam and so on. Exo encodes 5'-3' exonuclease, bet encodes overhang binding protein which enables annealing and recombination with complementary DNA, and gam encodes inhibitor of *E.coli* exonucleaseto protect introduced DNA. SW105 *E.coli*carries L-arabinose inducible *flp* gene, which encodes recombinase allow DNA modification without restriction enzyme and DNA ligase [21].

Cre-Lox recombination is a [site-specific recombinase technology](http://en.wikipedia.org/wiki/Site-specific_recombinase_technology) widely used to carry out deletions, insertions, translocations and inversions in the DNA of cells. It allows the DNA modification to be targeted to a specific cell type or be triggered by a specific external stimulus [22, 23]. For mouse line with Gpm6a GFPCreERT2 and ReelinGFPCreERT2, once the specific genes such as Gpm6a or Reelin begin to express, the GFPCreERT2 will express in the specific cell. Using GFP as a marker, we can trace the specific cells. Using CreERT2 system, we can knock out the specific gene in specific cell, which is conditional knockout [24].

For the next plan, we will Label hepatic mesothelial and hepatic stellate cells in the Gpm6a/ReelinGFPCreERT2 transgenic mouse, to trace the lineage of hepatic stellate cells. We also will make conditional TGFβ-KO mouse through crossover between TGFflox/flox mouse and Gpm6a/ReelinGFPCreERT2 mouse, to explore the function of hepatic stellate cells during the development.

**comments**

***Background***

Hepatic stellate cells (HSCs) activation is considered a major mechanism in the formation of fibrosis and cirrhosis. However fundamental questions concerning cell fate regulation of HSCs remain largely underexplored. Recent study reported that hepatic mesothelial cells are the potential precursors for HSCs in the development of liver and can transdifferentiate into myofibroblast cells in mouse liver fibrosis. Up to now, there are few specific mouse lines which can cause recombination (Cre) for tracing hepatic mesothelial cells or hepatic stellate cells (HSCs). Here, we describe a rapid and reliable strategy for construct preparation using bacterial artificial chromosome (BAC).

***Research frontiers***

Recent study reported that hepatic mesothelial cells are the potential precursors for HSCs in the development of liver and can transdifferentiate into myofibroblast cells in mouse liver fibrosis. Glycoprotein M6a (Gpm6a) has been identified as a specific surface marker of hepatic mesothelial cell, which covers on the surface of liver and migrates from surface into the center. Reelin is extracellular matrix glycoprotein, which is a specific HSCs marker in mouse liver and has similar amounts in resting and activated HSCs.

***Innovations and breakthroughs***

Up to now, there are few specific mouse lines which can cause recombination (Cre) for tracing hepatic mesothelial cells or hepatic stellate cells (HSCs). Here, the authors describe a rapid and reliable strategy for construct preparation using bacterial artificial chromosome (BAC). This study prepared for the first time Gpm6a/ReelinGFPCreERT2 construct, which is the first step for the preparation of Gpm6a GFPCreERT2 or ReelinGFPCreERT2 mouse line.

***Applications***

In this study, the construct of Gpm6a GFPCreERT2 or ReelinGFPCreERT2 was prepared successfully, which will establish foundation for tracing the hepatic stellate cells lineage and studying its function.

***Terminology***

Cre-Lox recombination is a [site-specific recombinase technology](http://en.wikipedia.org/wiki/Site-specific_recombinase_technology) widely used to carry out deletions, insertions, translocations and inversions in the DNA of cells. It allows the DNA modification to be targeted to a specific cell type or be triggered by a specific external stimulus. For mouse line with Gpm6a GFPCreERT2 and ReelinGFPCreERT2, once the specific genes such as Gpm6a or Reelin begin to express, the GFPCreERT2 will express in the specific cell. Using GFP as a marker, we can trace the specific cells. Using CreERT2 system, we can knock out the specific gene in specific cell, which is conditional knockout.

***Peer-review***

This is a good study in which the author prepared the construct of Gpm6a GFPCreERT2 or ReelinGFPCreERT2 successfully, which is the first step for the preparation of Gpm6a GFPCreERT2 or ReelinGFPCreERT2 mouse line. Cre mouse lines are very useful tools, which can generate knockout mice through the crossover between Cre and flox mouse lines.

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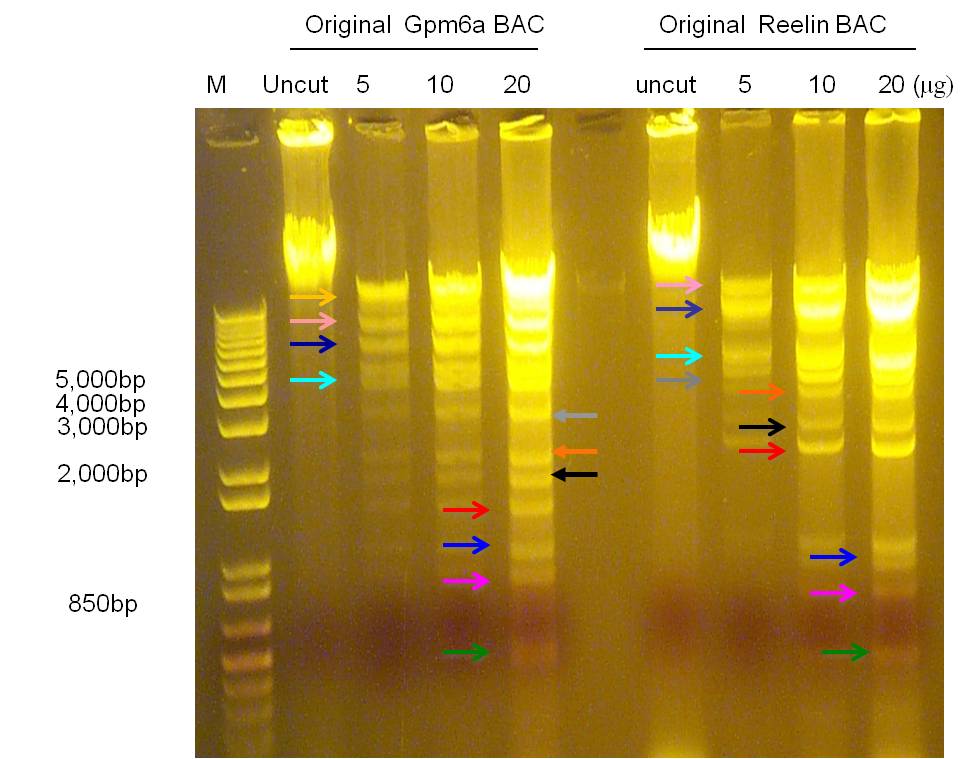
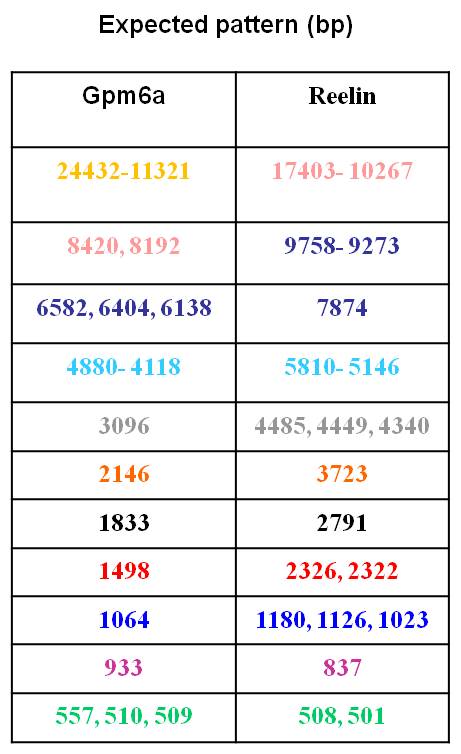
Grade C (Good): C

Grade D (Fair): 0

Grade E (Poor): 0

**Table 1 Primer sequence of Gpm6a and Reelin**

|  |  |  |
| --- | --- | --- |
|  | **Forward (F)** | **Reverse (R)** |
| Gpm6a 3arm | CGG TAC CTT TCA TGT TTT CAT GGT TGT CA | AGG TAC CGG CCG GCC ATG ACA GCA AAC ACT GCC TCT A |
| Gpm6a 5arm | ACC CAA TCT CCC TTT CAG | TGA ACT TGT GGC TTT AGA TC |
| Gpm6a Cre | ACC TGA AGA TGT TCG CGA TTA TCT | ACC GTC AGT ACG TGA GAT ATC TT |
| Reelin 3arm | AGG TAC CAC GGC ATC CCT ACG GCG C | AGG TAC CGG CCG GCC ACA GCC GCT CTG TTT CTT GAG G |
| Reelin 5arm | ACC CAA TCT CCC TTT CAG | TGA ACT TGT GGC TTT ACG TC |
| Reelin Cre | ACT TAA GCT CGT TCG CGC AGC G | AGT CGA CGC CGC CGC GCT CCG T |

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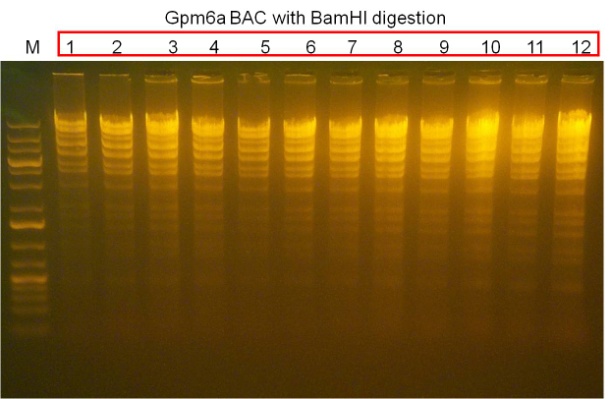
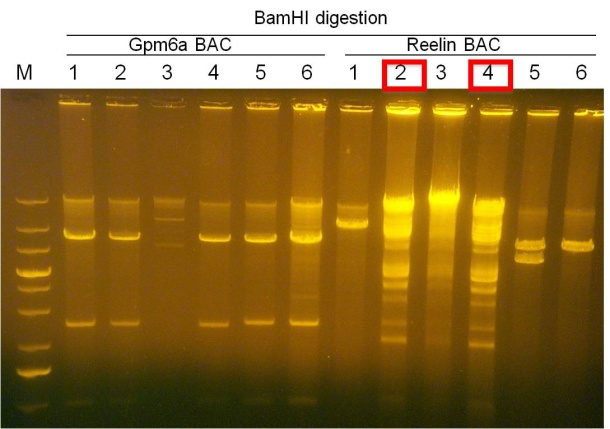
B

A

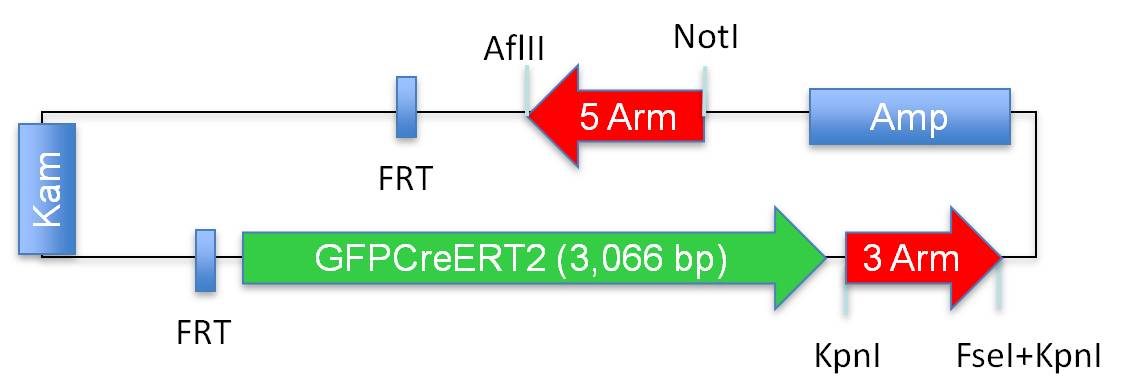
**Figure 1 Confirmation of BAC DNA with restriction enzyme digestion.** A: The electrophoretogram of original BAC with BamHI digestion; B: The expected bands of original BAC with BamHI digestion. The color of number is same as the arrow. M: Maker; Uncut: original BAC without digestion; 5, 10, 20: 5 μg, 10 μg, 20 μg of original BAC with BamHI digestion.

B

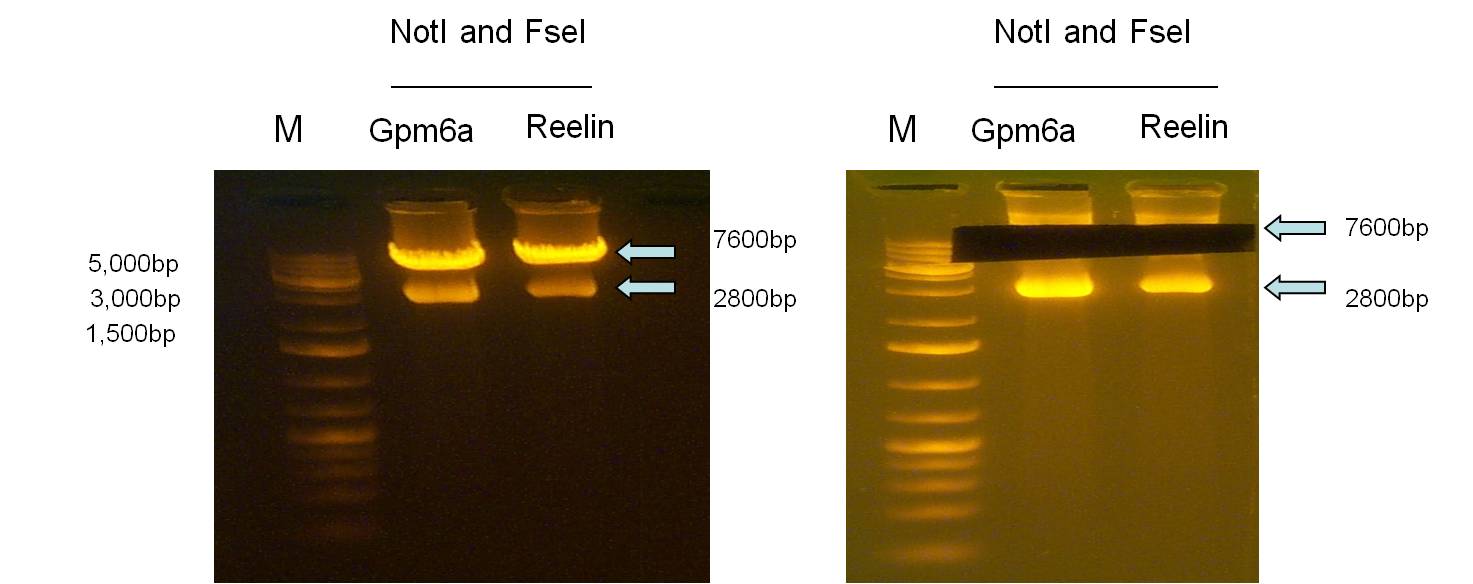
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**Figure 2 Confirmation of BAC DNA transformed into SW102 *E.coli.*** A: The electrophoretogram of Gpm6a and Reelin BAC purified from SW102 *E.coli* with BamHI digestion; B: The electrophoretogram of Gpm6a BAC with BamHI digestion. The positive clones were emphasized by red pane.

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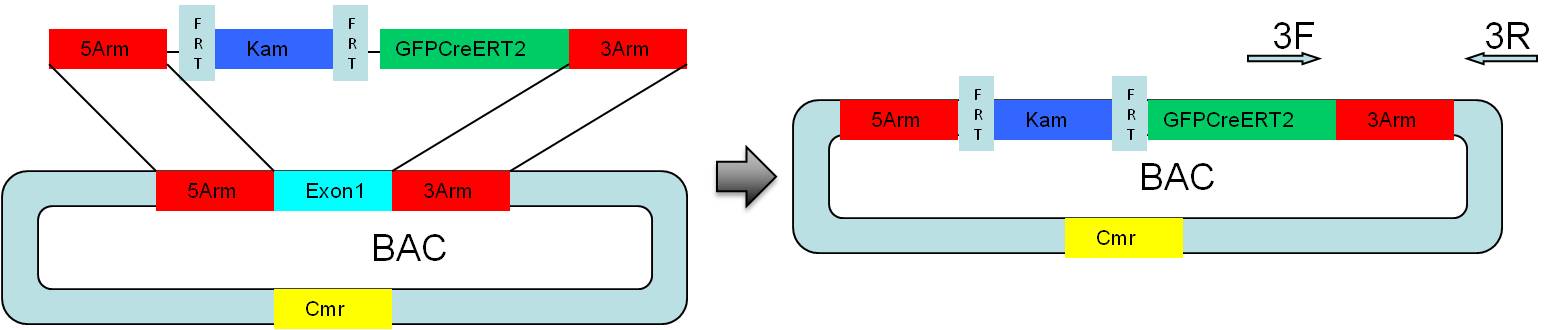
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C

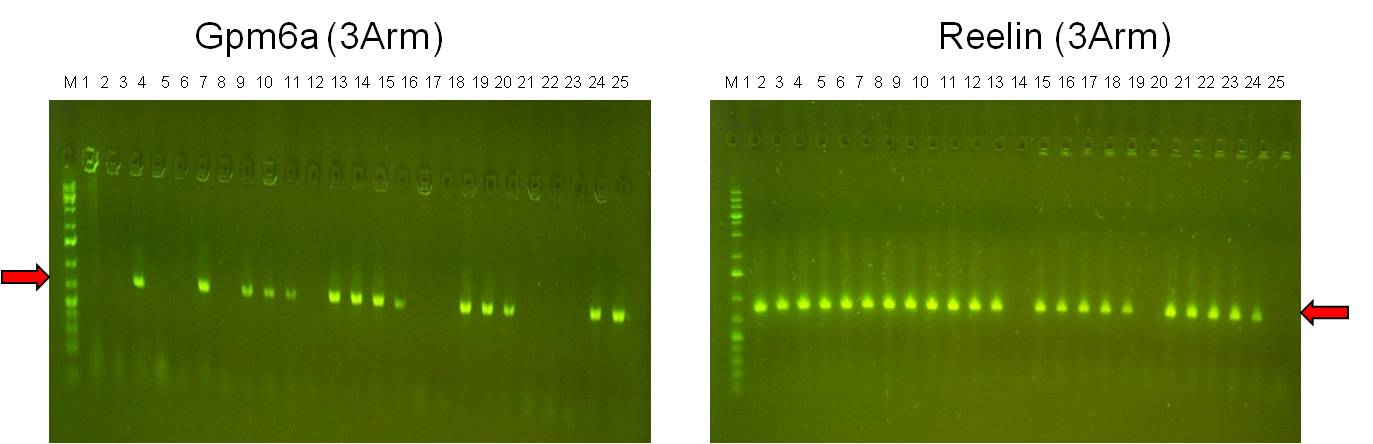
B

**Figure 3 Preparation of reporter gene GFPCreERT2.** A: The diagram of shuttle vector carrying GFPCreERT2; B: The electrophoretogram of the shuttle vector cut by NotI and FseI; C: The electrophoretogram after GFPCreERT2 fragment was cut from gel. M: Maker; Gpm6a: Shuttle vector inserted by 5 arm and 3 arm from Gpm6a BAC; Reelin: shuttle vector inserted by 5 arm and 3 arm from Reelin BAC.

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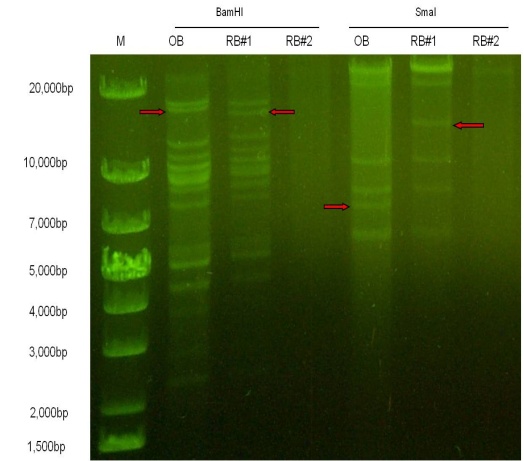
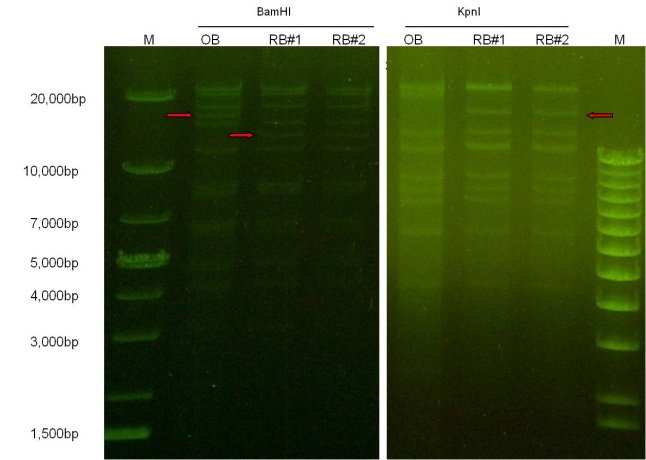
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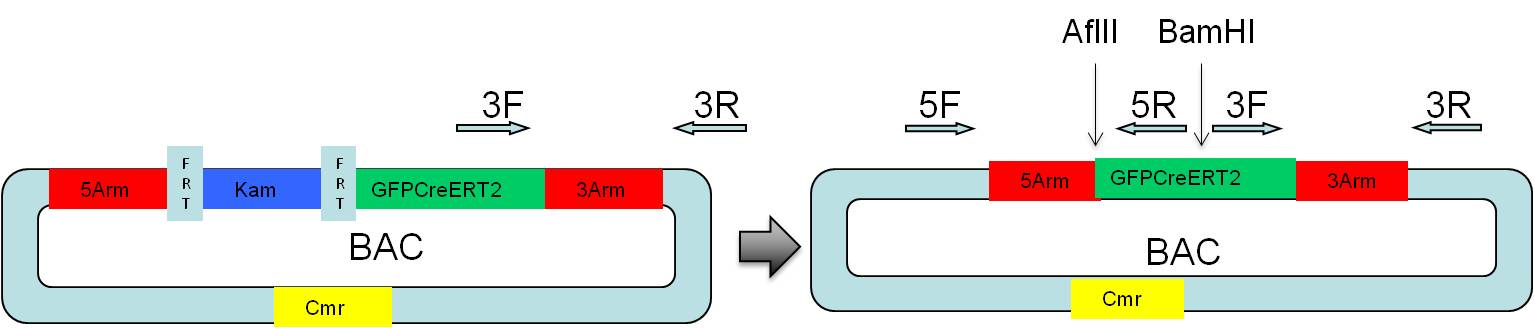
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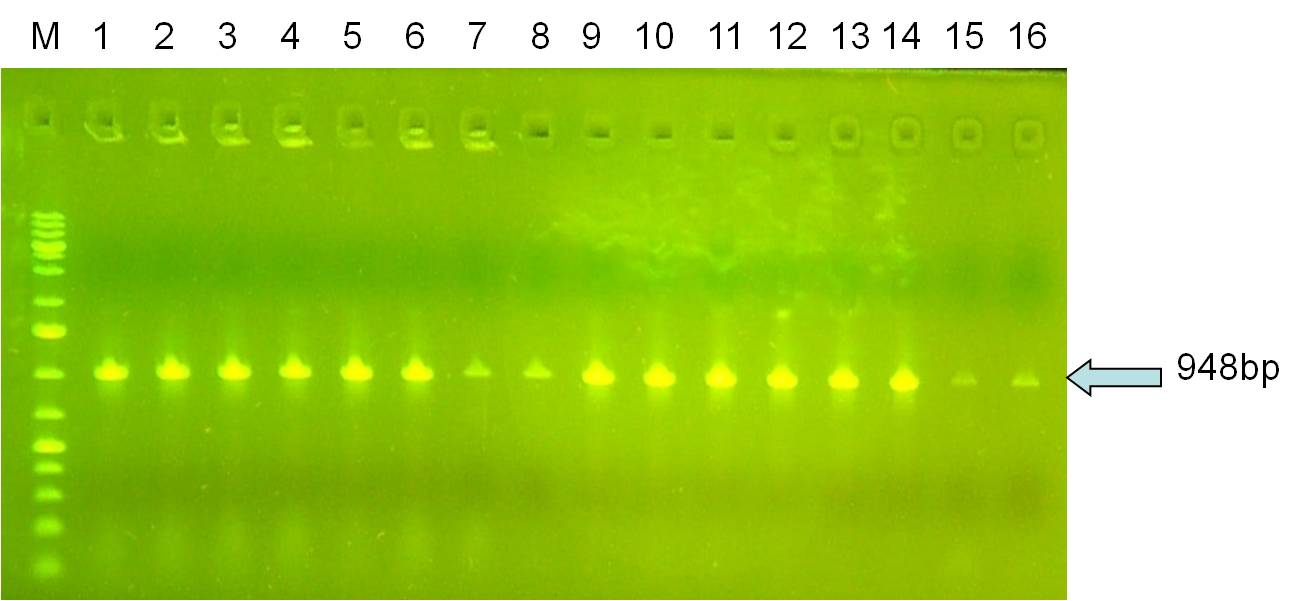
D

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**Figure 4 Screening and confirmation of recombinant clones.** A: The diagram of Homologous recombination between BAC and GFPCreERT2; B: The electrophoretogram of PCR product according to the recombinant BAC template with 3arm primer; C: The electrophoretogram of recombinant Gpm6a BAC digested by BamHI and KpnI. D: The electrophoretogram of recombinant Reelin BAC digested by BamHI and SmaI. M: Maker; OB: Original BAC; RB: Recombinant BAC.

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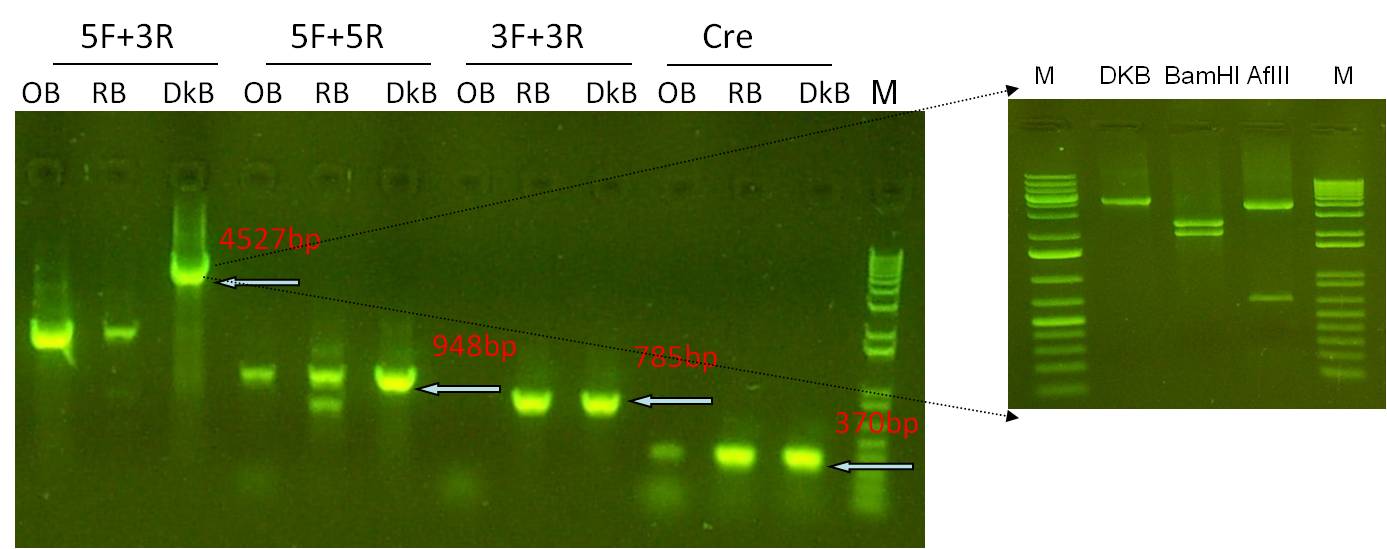
A

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B

D

C

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**Figure 5 Screening and confirmation of recombinant clones without kanamycin unit.** A: The diagram of Removal of Kam unit in SW105 *E.coli;* B:The electrophoretogram of PCR product according to the recombinant BAC template with 5arm primer; C: The electrophoretogram of PCR product according to the recombinant BAC template with different primers; D: The electrophoretogram of PCR product with BamHI or AflII digestion. M: Maker; OB: Original BAC; RB: Recombinant BAC; DkB: Deleted kanamycin BAC; 5F: Forward primer of 5arm; 5R: Reverse primer of 5arm; 3F: Forward primer of 3arm; 3R: Reverse primer of 3arm; Cre: Cre primer.