CHAPTER 3:
GENERATION OF THE *BCL11-LACZ* CONDITIONAL NULL REPORTER MICE

3.1 Introduction

3.1.1 Current *Bcl11* knockout mice

The original *Bcl11a* and *Bcl11b* knockout mice were reported by Pentao Liu (Liu et al., 2003b) and Yuichi Wakabayashi (Wakabayashi et al., 2003b) respectively. In each of these knockout mice, exon 1 of *Bcl11a* and *Bcl11b* was targeted and deleted using conventional gene targeting strategies. *Bcl11a* and *Bcl11b* heterozygous mutant mice were fertile and viable; however, homozygous mutant mice died within a few hours of birth from unknown reasons. Southern blot analysis of genomic DNA from homozygous mutant mice indicated the presence of the targeted alleles and Western blots showed expression of Bcl11a and Bcl11b only in the wild-type and heterozygous mutant mice, confirming that *Bcl11a* and *Bcl11b*-deficient mice were obtained. *Bcl11a* homozygous mutant embryos lacked B cells and had alterations in several T cells. Further studies suggested that Bcl11a functions upstream of Ebf1 and Pax5 in the B cell development pathway (Liu et al., 2003b). In contrast, *Bcl11b* homozygous mutant embryos showed a block at the $CD4^-CD8^-$ double-negative stage of thymocyte development without any impairment to B cell and γδ T cell lineages (Wakabayashi et al., 2003b). These results suggest that *Bcl11* genes are essential for lymphocyte development.

Exon 1 encodes the translation start site and the first 18 amino acids of both Bcl11a and Bcl11b. However, bioinformatical analysis of the *Bcl11* genomic regions showed that alternative transcriptional start sites exist within the first intron, downstream of exon 1. Semi-quantitative RT-PCR analysis of cDNA obtained from *Bcl11* homozygous mutant embryos detected the presence of transcripts that were derived from alternative transcriptional start sites (Personal communication with Pentao, Liu). These results suggest that the original *Bcl11* knockout mice are not complete null alleles but are severe hypomorphic alleles. Therefore, I decided to generate new null alleles for both *Bcl11a* and *Bcl11b* as part of the high-throughput recombineering vector construction

program (Chan et al., 2007). In contrast to exon 1, exon 4 of *Bcl11a* and *Bcl11b* encodes for at least 75% of the total protein-coding sequences of either Bcl11 proteins which contains the two main functional C_2H_2 zinc fingers. Therefore, to generate the *Bcl11-lacZ* conditional null reporter mice, I decided to target exon 4 of both *Bcl11* genes as it encodes the main functional domains of the proteins.

3.1.2 New recombineering reagents

Analysing knockout mice produced through gene targeting in mouse ES cell is still the most widely used approach to understand mammalian gene function. Knockout mutant lines have been generated for hundreds of mouse genes and analyses of these mutant lines have provided invaluable insights into mammalian gene functions (Austin et al., 2004). The first step in generating a knockout allele (null or conditional) is to construct a targeting vector in *E. coli* that is subsequently electroporated into mouse ES cells for homologous recombination. Our lab and others have previously described methods for constructing targeting vectors using recombineering, which is based on highly efficient homologous recombination systems from bacteriophages as detailed in Chapter 1.2.6 (Angrand et al., 1999; Lee et al., 2001; Liu et al., 2003a). A key bottleneck for genome-wide targeted mutagenesis programmes in the mouse is the generation of targeting constructs (Austin et al., 2004; Auwerx et al., 2004). We have recently developed a high-throughput recombineering system which can generate conditional knockout targeting vectors in a rapid and efficient manner (Chan et al., 2007). A key reagent for this new system includes a complete λ phage that is replication-defective in BAC-harbouring DH10B *E. coli* but still retains its heat-inducible homologous recombination functions. Another reagent includes a set of low-copy plasmids (pSim) that contains the genes encoding for recombineering functions in their native operon, *pL* under the control of λ CI repressor (Datta et al., 2006). By using cells containing either of these reagents, thousands of BAC clones can be made recombineering-competent either by transfection (λ phage) or transformation (pSim). Further improvement of recombineering in these systems allowed the steps for making targeting vectors to be performed in 96-well plates, making the whole process suitable for high-throughput operations. I decided to generate *Bcl11a-lacZ* and *Bcl11b-lacZ* reporter conditional null

mice using the new recombineering reagents in order to obtain (1) reporter alleles to study the expression patterns of these genes in other tissues and (2) exon 4 null alleles of *Bcl11* genes.

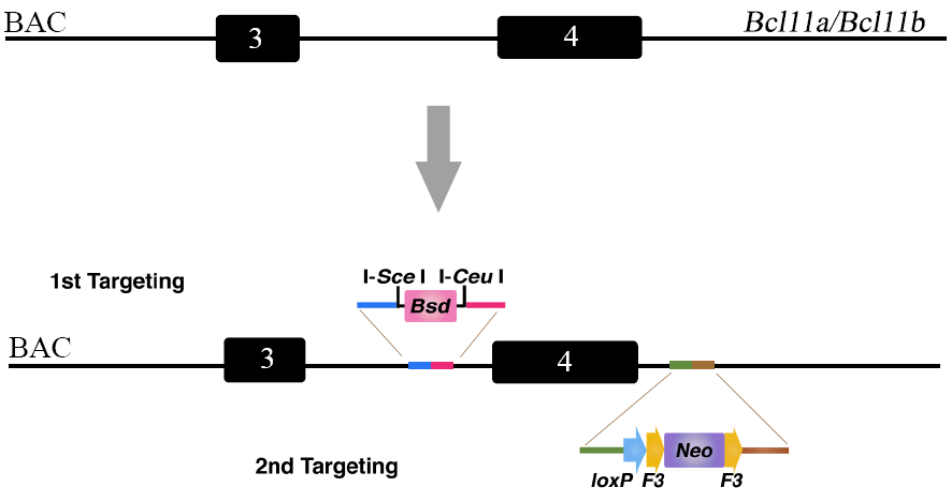
3.1.3 Targeting strategy for generating *Bcl11-lacZ* reporter alleles

The overall strategy for the construction of targeting and retrieval vectors for *Bcl11a* and *Bcl11b* is shown in Figure 3.1. Firstly, the BAC clones containing the *Bcl11a* and *Bcl11b* genomic regions were identified and verified. These BAC clones were then made recombineering-competent either by λ phage transfection or by transformation of pSim plasmids. Next, in order to generate targeting vectors with homologous sequences to the targeted region, PCR reactions using long primers were carried out using *Bsd*/*Neo*/retrieval cassettes as templates. Each primer used in the PCR reactions was 100 mers long, consisting of 80 bases of homologous sequence to the targeted region and 20 bases of homologous sequence to the *Bsd*/*Neo*/retrieval cassette templates. For both *Bcl11a-lacZ* and *Bcl11b-lacZ* targeting vectors, ~5-kb and ~3 kb of genomic DNA were chosen as the left and right homology arms respectively. The genomic DNA region to be deleted is 4,565 bp and 3,615 bp for *Bcl11a* and *Bcl11b* respectively and deletion would result in a frameshift. The PCR products were then purified and used for targeting to the BAC clones using recombineering. The *Bsd* cassette was first targeted to the BAC clones and successful transformants were selected using LB agar containing blasticidin (Bsd; Figure 3.1A). The Bsd-resistant (Bsd^R) BAC clones were then verified by checking for their resistance to tetracycline (Tet^R) to ensure that they had retained recombineering-competence before the second round of targeting. The *Neo* cassette containing *lox*P-*F3-PGK-EM7-Neo-F3* was then targeted to the BAC clones using recombineering and successful transformants were selected on LB agar containing kanamycin (Kan; Figure 3.1A). The Bsd^R and Kan-resistant (Kan^R) BAC clones were again verified to ensure that they still retained recombineering-competence. Next, the correctly *Bsd*- and *Neo*-targeted BAC DNA were retrieved into the retrieval plasmid PL611 using recombineering and the successfully retrieved clones were selected on LB agar containing ampicillin (Amp; Figure 3.1B). The Bsd^R–Kan^R–Amp^R (Amp-resistant) retrieval plasmids were then cut with two rare restriction homing endonucleases *I-Sce*I and *I-Ceu*I and ligated with a

similarly cut *lacZ-Puro* cassette to generate the *Bcl11a-lacZ* and *Bcl11b-lacZ* targeting constructs (Figure 3.1C). Finally, the negative selection marker Thymidine kinase (*TK*) with the chloramphenicol acetyltransferase gene (*Cm*) was added to the backbone of the retrieval vector by recombineering; replacing the Amp^R coding sequence. The final *Bcl11a-lacZ* and *Bcl11b-lacZ* reporter targeting constructs were then verified by restriction digestion and sequencing before targeting to ES cells.

The flexibility of these targeting vectors enables the generation of a multi-purpose allele that can serve as a reporter, knockout and a conditional knockout allele. The expression of *lacZ* is driven by the endogenous *Bcl11* regulatory elements, functioning as a reporter allele (Figure 3.1D). By staining $Bcl11^{lacZ/+}$ heterozygous embryos or tissues with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), *Bcl11* expression can be detected by the presence of blue staining. To generate a conditional knockout allele, the *lacZ* reporter mouse line can be crossed to a ubiquitous Flpe recombinase-expressing mouse line to excise the *lacZ-Puro* and the *Neo* cassettes. Upon expression of Flpe recombinase, the intervening sequences between the respective *FRT* and *F3* sites are deleted, leaving behind two *lox*P sites flanking exon 4 of *Bcl11a* and *Bcl11b*, generating a conditional knockout allele (Figure 3.1E). Subsequently, the mouse line with the conditional knockout allele can be crossed to a tissue-specific Cre recombinase-expressing mouse line. Upon expression of Cre recombinase, the intervening sequences between the two *lox*P sites would be deleted, producing a null allele (Figure 3.1F).

A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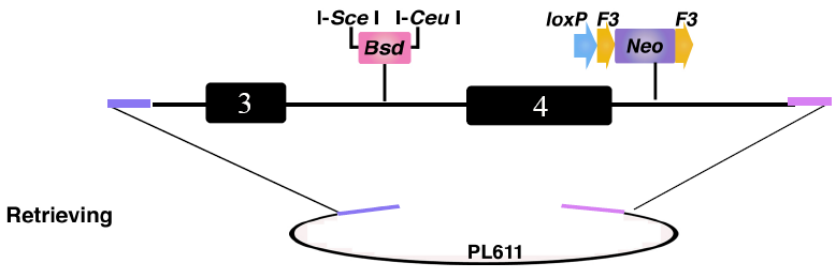
3
4
Bcl11a/Bcl11b
1st Targeting
I-Sce I
I-Ceu I
Bsd
BAC
3
4
2nd Targeting
Neo
loxP
F3
F3

B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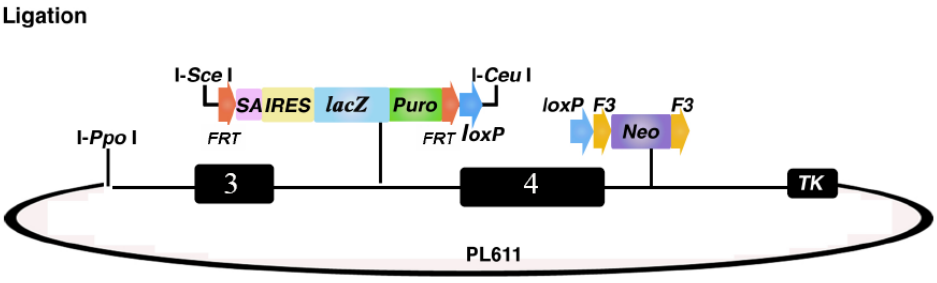
I-Ceu I
Bsd
loxP
F3
F3
Neo
3
4
Retrieving
PL611

C


Ligation
I-Sce I
I-Ceu I
SA
IRES
lacZ
Puro
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F3
F3
Neo
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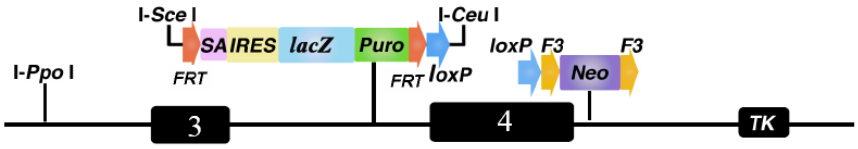
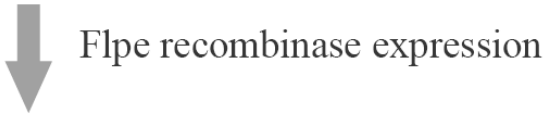
loxP
3
4
TK
PL611

D





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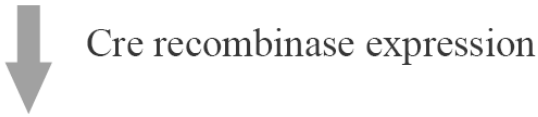


F

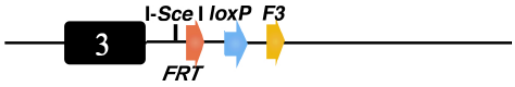


Figure 3.1. Construction of conditional knockout targeting vectors using the new recombineering reagents. (A) The genomic structure of the *Bcl11* locus with exon 4 to be deleted in the conditional knockout (cko) allele. The *Bsd* cassette flanked by two rare cutter sites, *I-Sce*I and *I-Ceu*I, is targeted to the 5' side of the intended deletion region. Subsequently, the *lox*P-*F3-PGK-EM7-Neo-F3* (*Neo*) cassette is targeted to the 3' side of the deletion region. Coloured lines represent the short homology arms used for recombineering. **(B)** The genomic DNA fragment is then retrieved from the BAC to PL611, which has the Amp^R gene. **(C)** The *Bsd* cassette can be conveniently replaced by a reporter, i.e. *lacZ*, in a simple ligation reaction. The final targeting vector has the reporter flanked by two *FRT* sites followed by a *lox*P site at the 5' side of the intended deletion region, and a *F3* flanked *Neo* cassette providing positive selection in ES cells. The negative selection marker *TK* is added to the vector backbone by recombineering. **(D)** The vector is then linearized with the rare-cutter *I-Ppo*I before targeting to ES cells. **(E)** Expression of Flpe recombinase would lead to recombination between the specific *FRT/F3* sites, resulting in removal of the *lacZ-Puro* reporter (*FRT*) and the *Neo* cassette (*F3*) sequences, leaving behind a single *FRT* and *F3* site at the 5' and 3' end of exon 4. This creates the cko alleles for *Bcl11a* and *Bcl11b* as exon 4 of either gene is now flanked by *lox*P sites and deletion of this exon can be mediated by tissue-specific expression of Cre recombinase. **(F)** Expression of Cre recombinase would lead to recombination between the two *lox*P sites and result in deletion of the intervening sequences (including exon 4 of each *Bcl11* gene), leaving behind a single *lox*P site. (Modified from Chan *et al.;* 2007).

3.2 Results

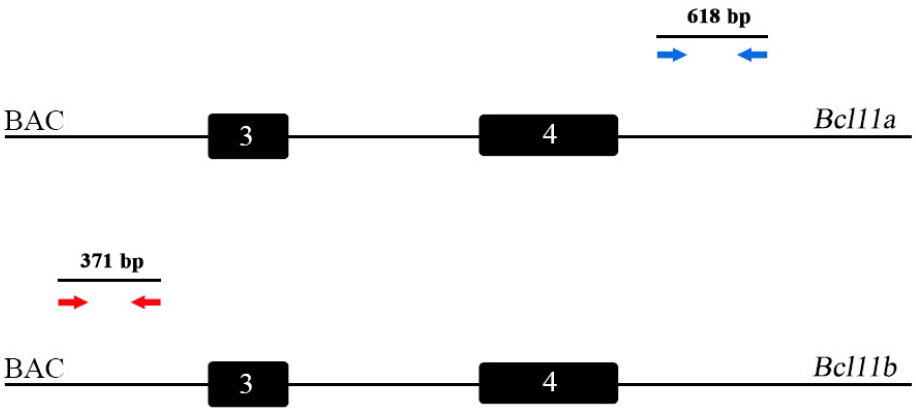
3.2.1 Construction of targeting and retrieval vectors

BAC clones containing the *Bcl11a* (bMQ-202K13 and bMQ-44A03) and *Bcl11b* (bMQ-188F04) genomic regions were identified from Wellcome Trust Sanger Institute 129/AB2.2 BAC library. BAC DNA were extracted and verified with sequence specific primers to ensure that they indeed contained the genomic regions of interest (Figure 3.2A). Next, templates for the PCR reactions were generated by restriction digestion of the *Bsd*/*Neo*/retrieval plasmids with appropriate enzymes and purification of the fragments of interest (Figure 3.2B-D). PCR reactions were then carried out using 1 to 5 ng of template (purified *Bsd*/*Neo*/retrieval cassettes) and PAGE-purified primers. The PCR products were detected in a 1% agarose gel and the appropriate bands purified to obtain the recombineering substrates (Figure 3.2E-G). The verified BAC clones were either transfected with λ phage (strain LE392) or transformed with pSim18 to deliver recombineering-competence. Transformants were selected on LB agar containing Tet (λ phage) or Hygromycin (Hyg; pSim18). Typically, there were between 500 to 1000 Tet^R (Tet-resistance) or Hyg^R (Hyg-resistance) colonies obtained for each BAC clone. One Tet^R or Hyg^R BAC clone was then used for the first targeting with the *Bsd* PCR product and successful transformants were selected on LB agar containing Bsd. There were between 20 to 100 Bsd^R colonies obtained for each BAC clone. Two Bsd^R colonies were picked for each clone and streaked onto LB agar containing Amp to determine if Bsd^R was due to contamination of the uncut *Bsd* cassette. None of the clones were Amp^R, suggesting that these Bsd^R clones were derived from successful *Bsd* cassette targeting. Next, the Bsd^R clones were checked for their susceptibility to Tet or Hyg to determine if the BAC clones had retained their recombineering competence after the first round of targeting. None of the Bsd^R clones were Tet^R or Hyg^R, suggesting that they had lost their recombineering competence. These Bsd^R clones were again either transfected with λ phage or transformed with pSim18 to restore recombineering-competence. Successful transformants were used for the next round of targeting.

After targeting of the *Bsd* cassette to the BAC DNA, the *Neo* PCR product, which is flanked by two *F3* sites and one *lox*P site, was targeted to the 3' side of the deletion region. *F3* is a mutant *FRT* site and in the presence of Flpe recombinase, recombination

occurs only between two *F3* sites or wild-type *FRT* sites but not between *F3* and *FRT* sites (Schlake and Bode, 1994). The *Neo* cassette is driven by a *PGK* promoter and an *EM7* promoter and is therefore functional in both mammalian cells and *E. coli*. There were between 50 to 100 Kan^R colonies obtained for each BAC clone. Two Kan^R colonies were picked for each clone and tested for their susceptibility to Amp and Bsd. All the clones were Bsd^R but susceptible to Amp, suggesting that these clones were derived from successful *Bsd* and *Neo* cassette targeting. Following completion of the two targeting steps, the modified BAC was subsequently retrieved to a modified pBR322 plasmid (PL611). pBR322 replication origin was used instead of pUC19 to reduce the instability problems associated with cloning mouse genomic DNA into high-copy pUC-typed plasmid. There were between 10 to 50 Amp^R colonies obtained from this round of retrieving.

A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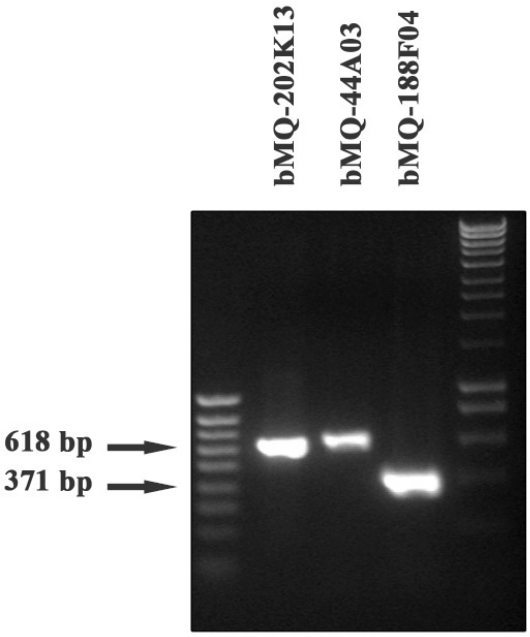
BAC
3
4
Bcl11a
371 bp
BAC
3
4
Bcl11b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371 bp

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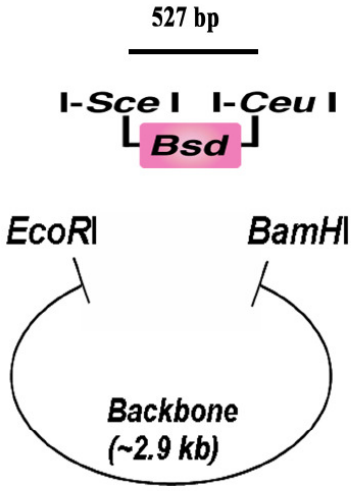
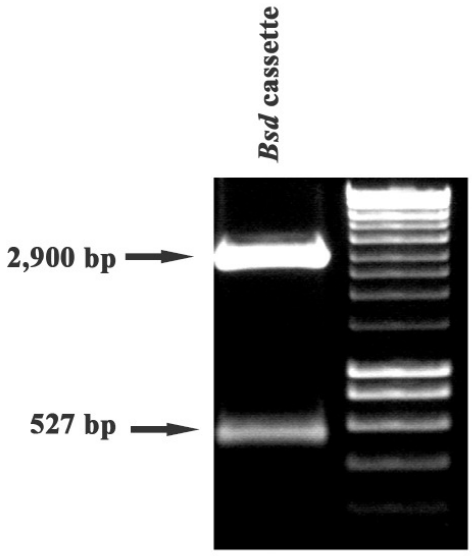
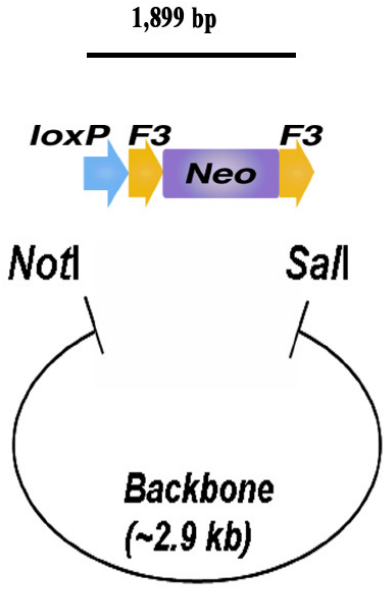
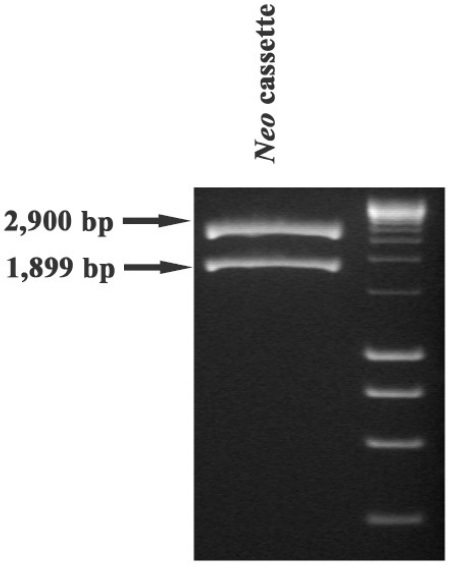

527 bp
I-Sce I
I-Ceu I
Bsd
EcoRI
BamHI
Backbone
(~2.9 kb)

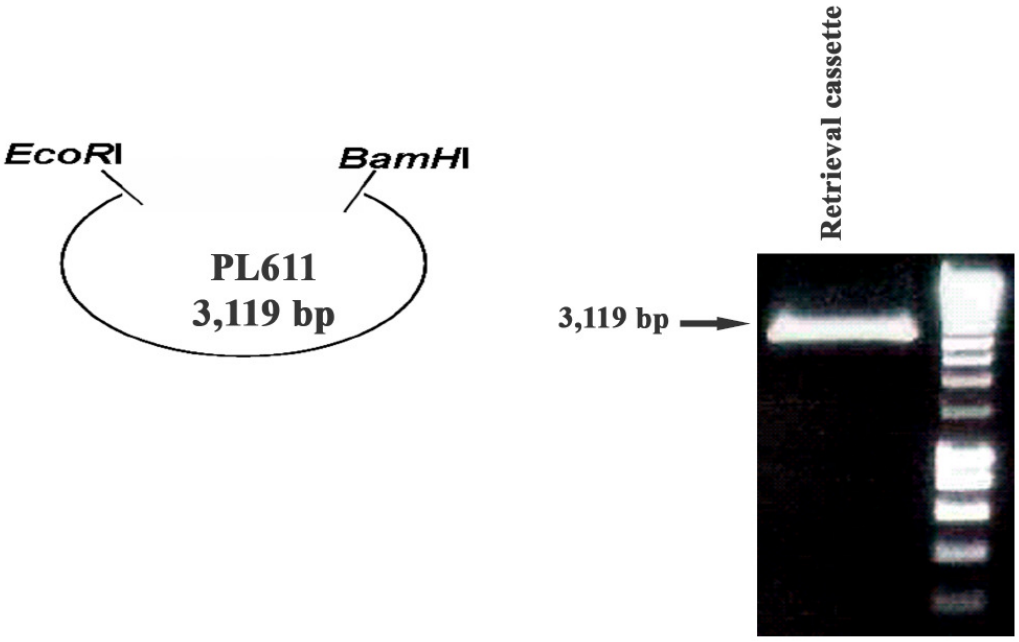

Bsd cassette
2,900 bp
527 bp

C

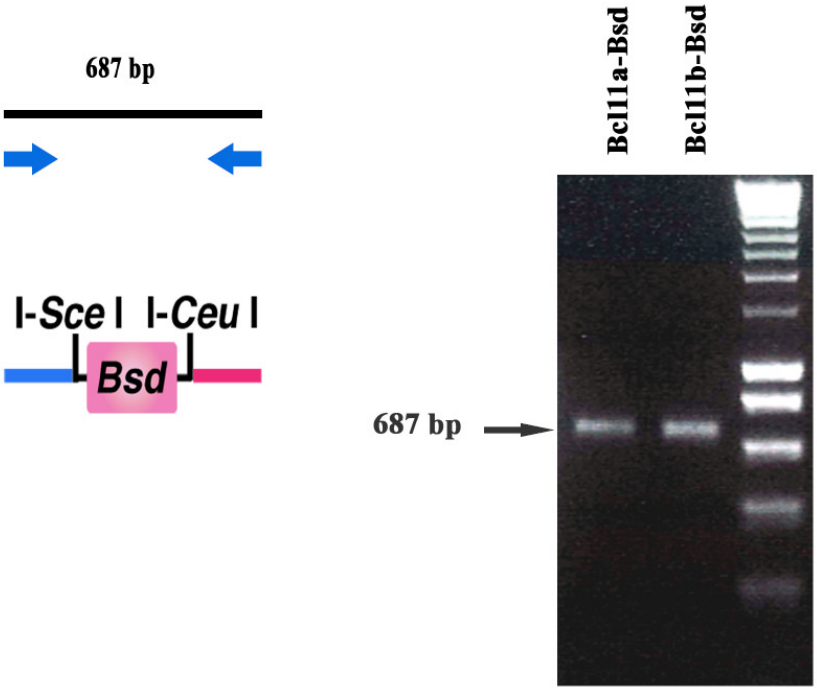

1,899 bp
loxP
F3
Neo
F3
NotI
SalI
Backbone
(~2.9 kb)


Neo cassette
2,900 bp
1,899 bp

D


EcoRI
BamHI
PL611
3,119 bp
Retrieval cassette
3,119 bp

E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Bsd
Bcl11a-Bsd
Bcl11b-Bsd
687 bp

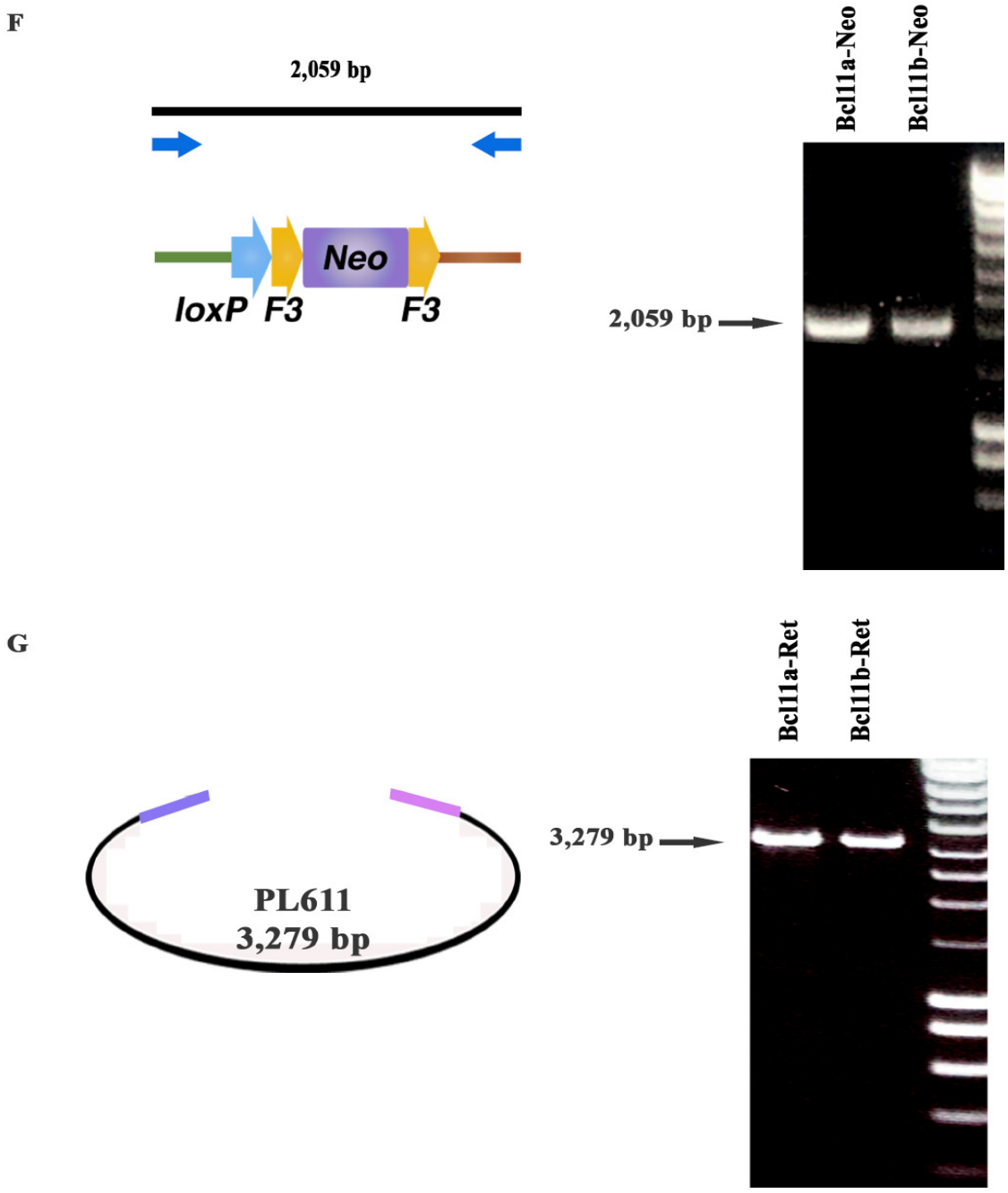


Figure 3.2. Construction of recombineering substrates by PCR. (A) Schematic diagrams depicting positions of primers used to validate BAC clones. Gel image showing PCR validation of the *Bcl11a* (bMQ-202K13 and bMQ-44A03) and *Bcl11b* (bMQ-188F04) BAC clones by sequence-specific primers. Gel images showing digestion products of **(B)** *Bsd* **(C)** *Neo* **(D)** retrieval plasmids after restriction digestion with appropriate enzymes as indicated in schematic diagrams. Fragments corresponding to **(B)** 527 bp **(C)** 1,899 bp and **(D)** 3,119 bp of *Bsd*, *Neo* and retrieval (Ret) cassette are purified and used as templates for PCR reactions. Gel images showing PCR products of **(E)** *Bsd* (687 bp), **(F)** *Neo* (2,059 bp) and **(G)** Ret (3,279 bp) cassettes. Homology arms of each product are shown as coloured blocks. PCR products are purified and used for recombineering.

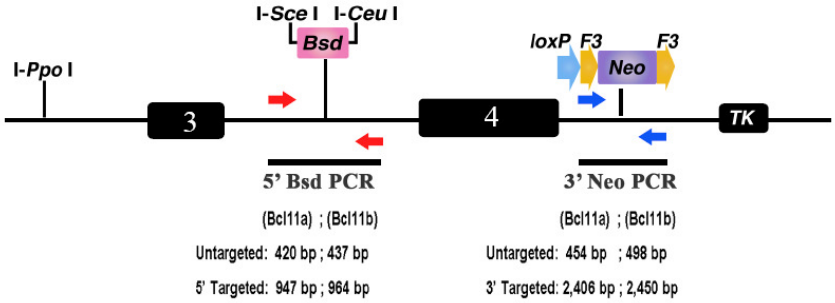
The retrieved doubly-targeted plasmids were then verified by pairs of gene-specific primers as shown in Figure 3.3A. Each of these primer pair were designed to span 200 to 250 bp either side of the 5' *Bsd* and 3' *Neo* targeted sites. Therefore, PCR amplification of plasmids containing non-modified genomic regions would produce a 420/454 bp (*Bcl11a/Bcl11b*) and/or a 437/498 bp (*Bcl11a/Bcl11b*) band respectively. In contrast, the BACs with correctly targeted 5' *Bsd* and 3' *Neo* cassettes would produce a 947/964 bp (*Bcl11a/Bcl11b*) and a 2,406/2,450 bp (*Bcl11a/Bcl11b*) band respectively (Figure 3.3A). As shown in Figure 3.3B, each of the two retrieved doubly-targeted plasmids contained the *Bsd* and *Neo* cassettes correctly targeted to the 5' and 3' regions.

Next, the 5' *Bsd* cassette was replaced by the *lacZ-Puro* reporter cassette. The *lacZ-Puro* cassette, with its splice acceptor (*SA*) and its polyadenylation site, would disrupt full transcription of the endogenous allele, creating a loss-of-function allele. Both the *Bsd* cassette and the *lacZ-Puro* cassette are flanked by two rare cutter sites, *I-Sce*I and *I-Ceu*I. Restriction digestion of the retrieved BAC clones with *I-Sce*I and *I-Ceu*I would result in the release of the 5' *Bsd* cassette (Figure 3.3C). The *lacZ-Puro* cassette was also restriction digested with *I-Sce*I and *I-Ceu*I (Figure 3.3D) and the purified fragment was ligated into the digested retrieved plasmids to generate the *Bcl11a-lacZ* and *Bcl11b-lacZ* targeting constructs. Selection of *E. coli* cells containing the *Bcl11a-lacZ* and *Bcl11b-lacZ* targeting constructs were done on LB agar containing Kan and Puro. Double antibiotic selection was used to eliminate any background. There were between 10 to 25 Kan^R and $Puro^R$ colonies obtained, usually after a 16 to 24 hour incubation period. Two Kan^R and $Puro^R$ colonies of each construct were picked and the plasmids were restriction digested with either *I-Ppo*I or *I-Sce*I in combination with *I-Ceu*I to confirm that they contained the *lacZ-Puro* insert. Figure 3.3E showed that all 4 clones contained the *lacZ-Puro* insert (6,814 bp).

Finally, the negative selection marker *MC1-TK* was targeted to the backbone of the *Bcl11a-lacZ* and *Bcl11b-lacZ* targeting constructs, replacing the Amp^R coding sequence. The *MC1-TK* serves as a negative selection marker for targeting in ES cells. This strategy relies on the principal that those cells in which random integration has occurred is likely to retain the *TK* gene and will thus be specifically eliminated by the antiviral agent gancyclovir (Thomas et al., 1986). By this means, enrichment in properly

targeted clones could be obtained. The *Cm-TK* cassette was restriction digested (Figure 3.3F) and the required fragment was purified and used to target *Cm-TK* to the backbone of the retrieved plasmids by recombineering. Successful transformants were selected on LB agar containing Kan and Cm. There were about 50 to 100 Kan^R and Cm^R colonies. Four Kan^R and Cm^R colonies from each construct were selected and tested for their resistance to Puro, Kan, Cm and Amp. Clones containing the final targeting constructs with *Cm-TK* were $Puro^R$, Kan^R, Cm^R but susceptible to Amp. All the selected clones were found to contain the correct final targeting constructs. The final targeting constructs were then sequenced to ensure that there were no mutations within the *lacZ* reporter, *lox*P, *FRT* and *F3* sequences.

A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I-Ceu I
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loxP
F3
F3
Neo
I-Ppo I
3
4
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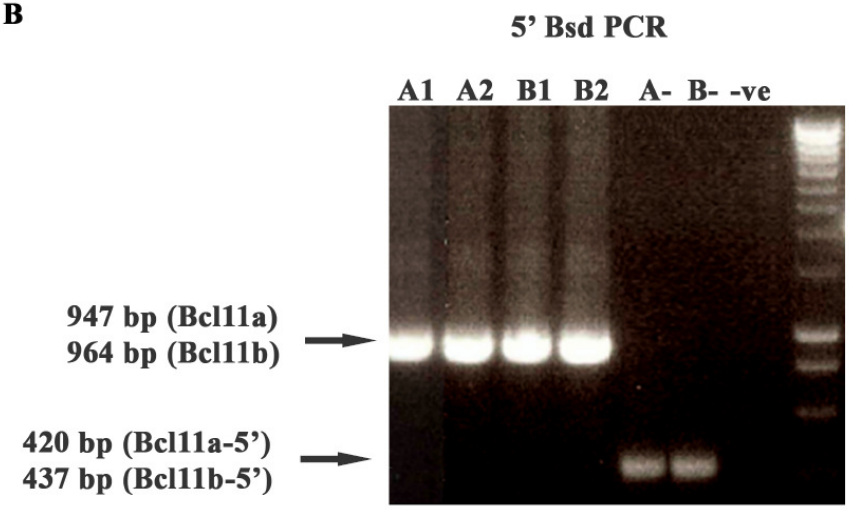
(Bcl11a) ; (Bcl11b)
Untargeted: 420 bp ; 437 bp
5' Targeted: 947 bp ; 964 bp
3' Neo PCR
(Bcl11a) ; (Bcl11b)
Untargeted: 454 bp ; 498 bp
3' Targeted: 2,406 bp ; 2,450 bp

B


5' Bsd PCR
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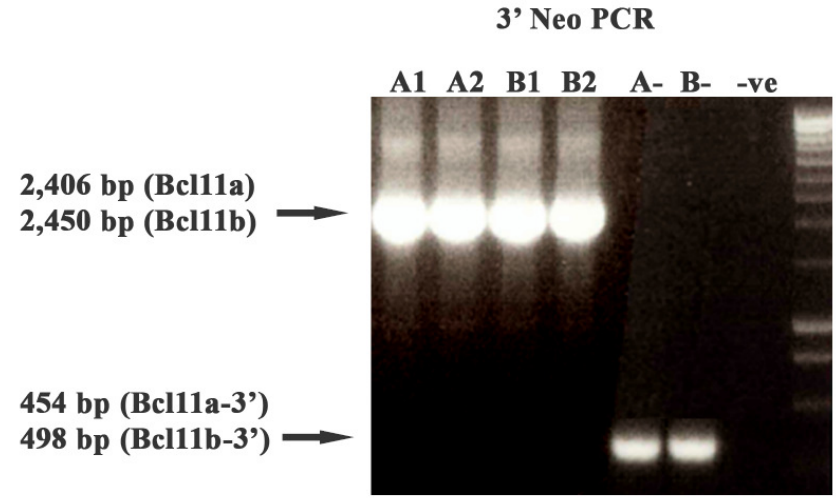
964 bp (Bcl11b)
420 bp (Bcl11a-5')
437 bp (Bcl11b-5')


3' Neo PCR
A1 A2 B1 B2 A- B- -ve
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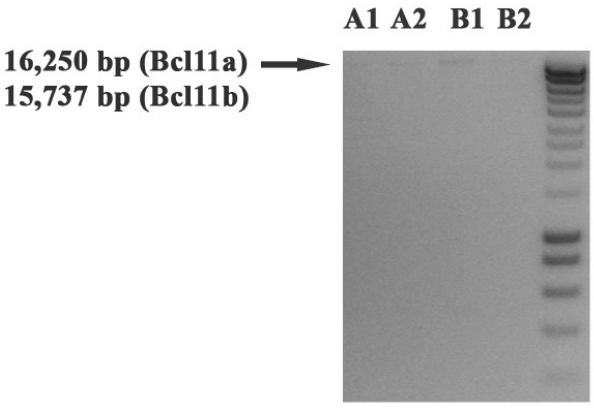
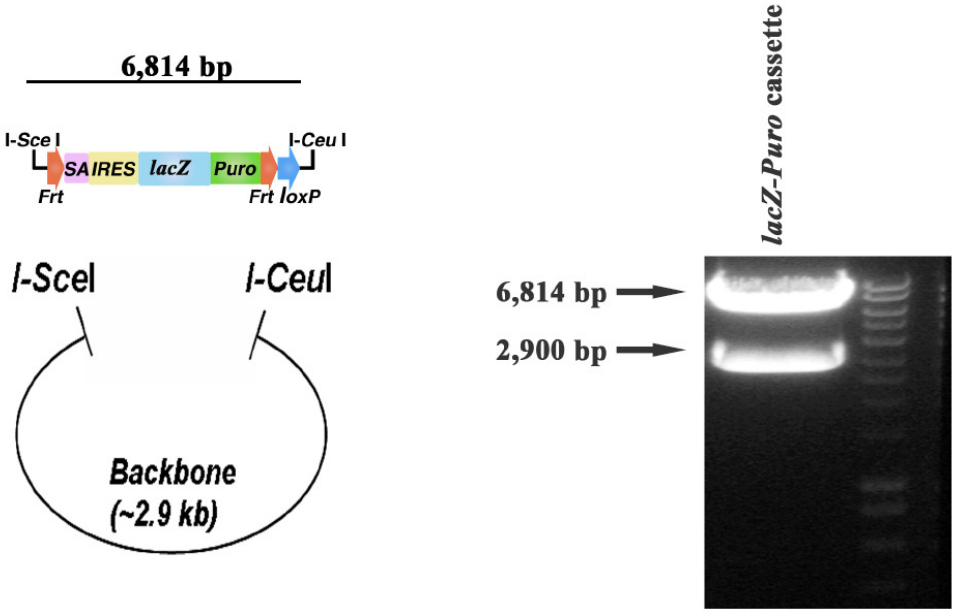
2,450 bp (Bcl11b)
454 bp (Bcl11a-3')
498 bp (Bcl11b-3')

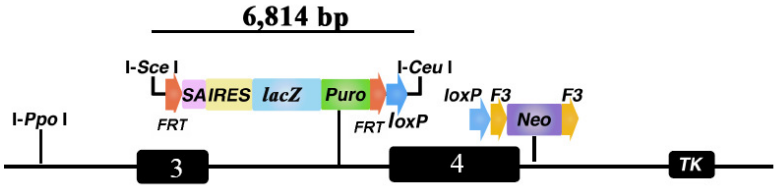
C

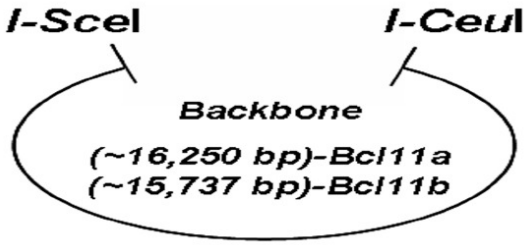

A1 A2 B1 B2
16,250 bp (Bcl11a)
15,737 bp (Bcl11b)

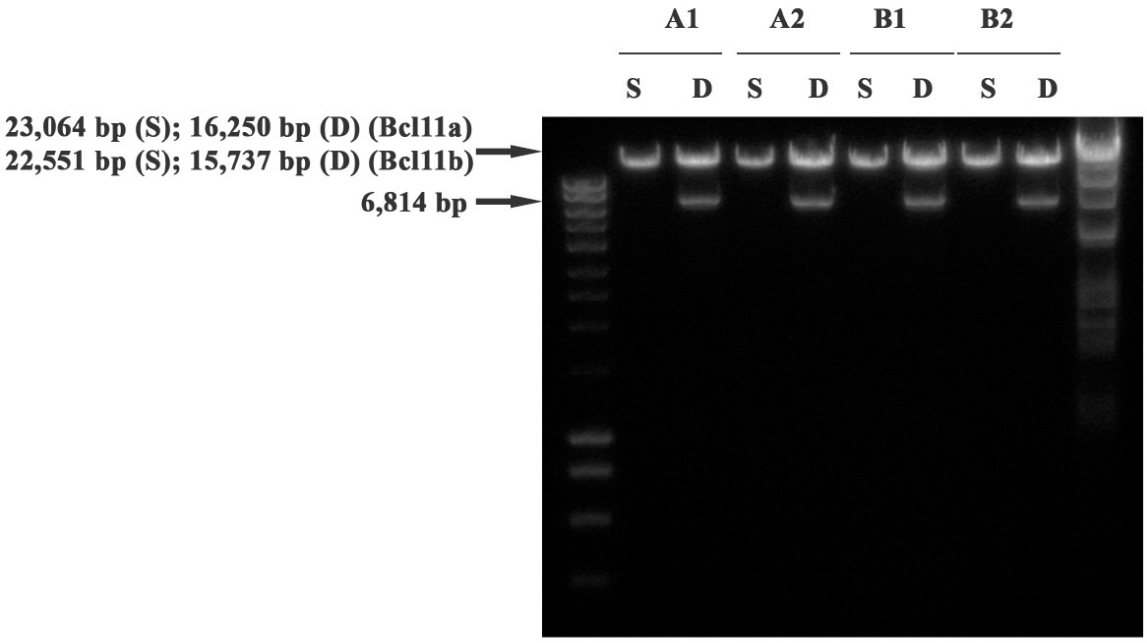
D


6,814 bp
I-Sce I
I-Ceu I
SA
IRES
lacZ
Puro
Frt
Frt
loxP
I-SceI
I-CeuI
Backbone
(~2.9 kb)
lacZ-Puro cassette
6,814 bp
2,900 bp

E







S: *I-Ppo*I digest

D: *I-Sce*I +*I-Ceu*I digest

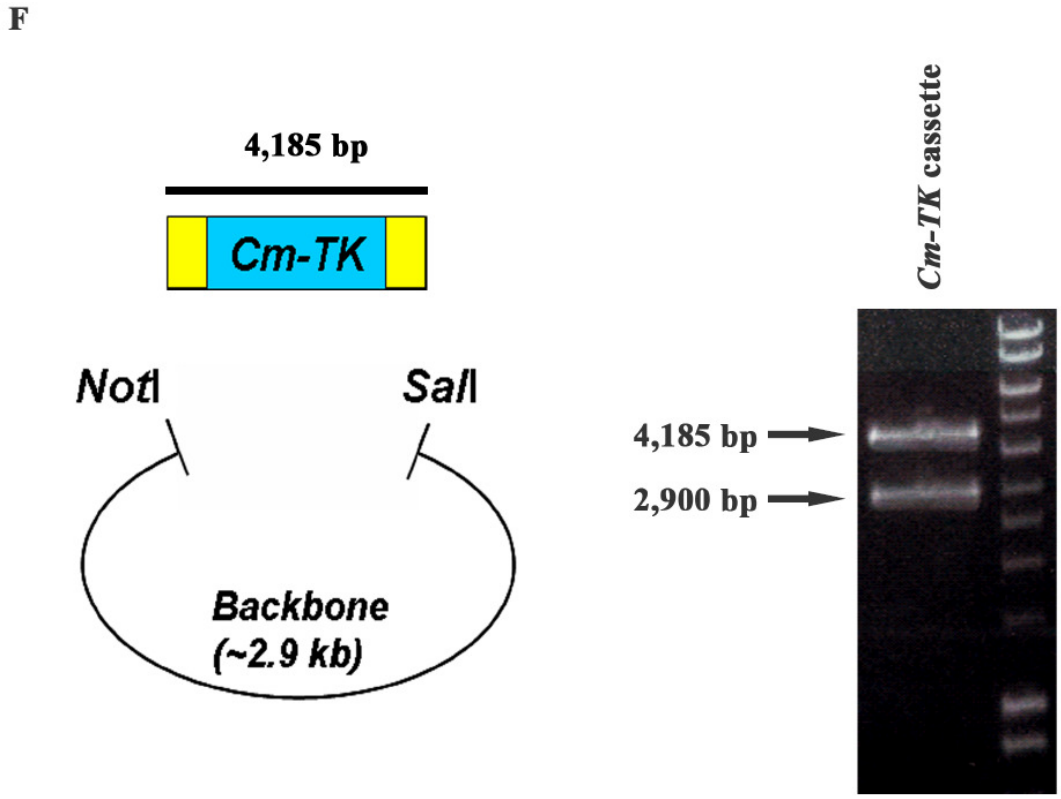


Figure 3.3. Validation of targeting constructs. (A) Red arrows indicate primer pairs for validating 5' *Bsd* targeting while blue arrows indicate primer pairs for validating 3' *Neo* targeting. Expected PCR products from untargeted and targeted regions are as shown in figure. **(B)** Gel image showing validation PCR products from *Bcl11a* (A1/A2) and *Bcl11b* (B1/B2) targeting vectors. *Bcl11a* constructs with successful *Bsd* and *Neo* targeting show 947 bp and 2,406 bp PCR products with *Bsd* and *Neo* validation primers respectively. Similarly, *Bcl11b* constructs with successful *Bsd* and *Neo* targeting show 964 bp and 2,450 bp PCR products with *Bsd* and *Neo* validation primers respectively. A-/B-/-ve indicate no template controls. **(C)** Restriction digestion confirmation of plasmids with successful retrieval of *Bsd* and *Neo* targeted *Bcl11a* and *Bcl11b* BAC regions. **(D)** Restriction digestion of *lacZ-Puro* reporter plasmids with *I-Sce*I and *I-Ceu*I produces a 6,814 bp fragment that contains the *lacZ-Puro* reporter. This fragment is purified and used for ligation with *Bcl11a* and *Bcl11b* targeting vectors that have been cut with *I-Sce*I and *I-Ceu*I. **(E)** Confirmation of *Bcl11a* and *Bcl11b* targeting vectors that have been successfully ligated with *lacZ-Puro* reporter cassette. Linearization of the targeting constructs with *I-Ppo*I produces a 23,064 bp band for *Bcl11a* and a 22,551 bp band for *Bcl11b*. In contrast, restriction digestion of the targeting constructs with *I-Sce*I and *I-Ceu*I results in the release of the *lacZ-Puro* insert (6, 814 bp) and a backbone of 16,250 bp and 15,737 bp for *Bcl11a* and *Bcl11b* constructs respectively. **(F)** Restriction digestion of *Cm-TK* plasmid with *Not*I and *Sal*I releases the *Cm-TK* insert (4,185 bp) from the pBluescript backbone. This fragment is purified and used for recombineering. Yellow areas denote homologous sequences to Amp^R sequence on PL611 backbone.

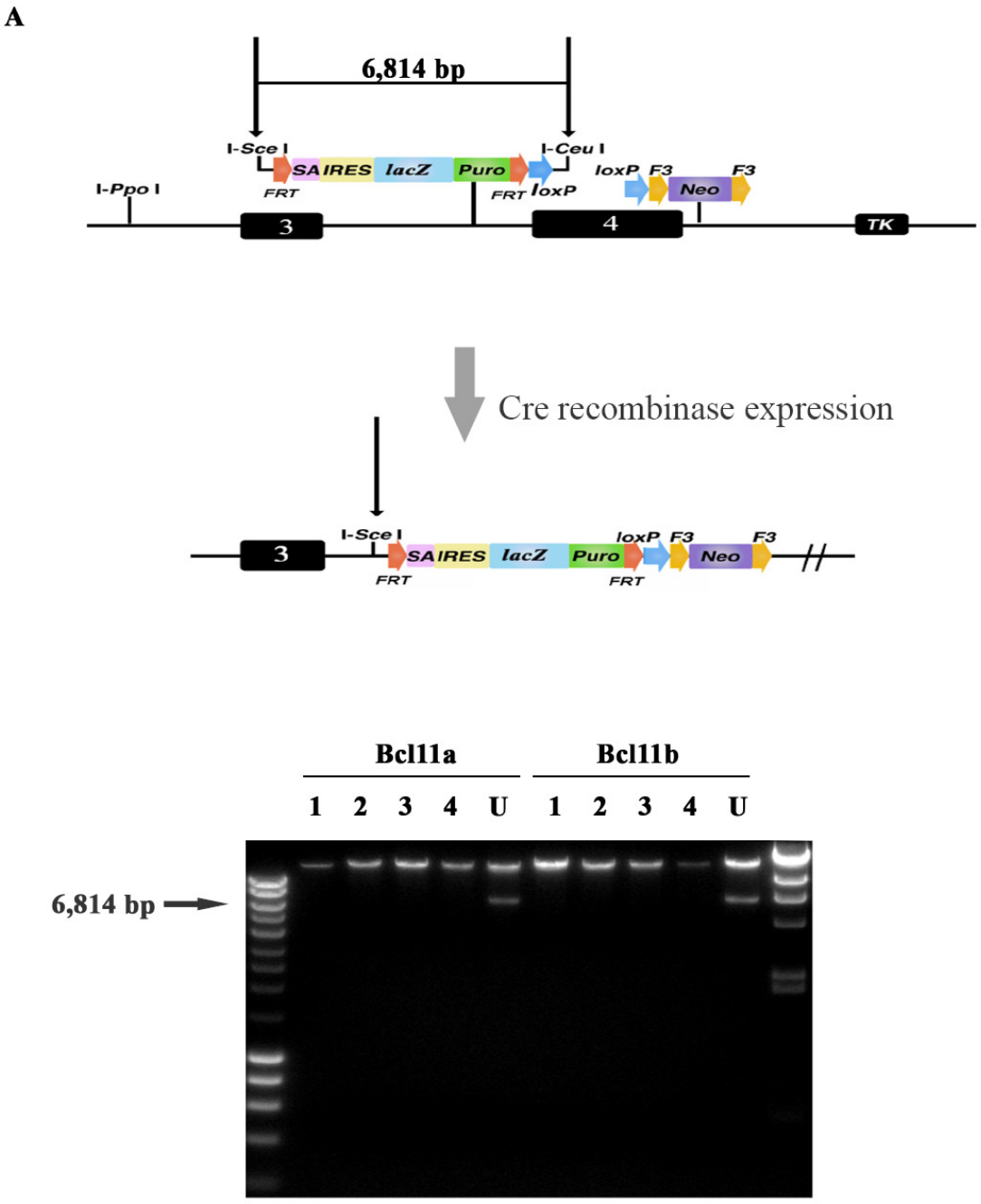
3.2.2 Verification of functionality of targeting constructs

The final targeting constructs described above would allow me to generate a flexible multi-purpose allele in the mouse. The *lacZ*-tagged alleles also serve as null alleles since the targeted *lacZ-Puro* cassette contains a splice acceptor (*SA*) which would disrupt endogeneous *Bcl11* transcription. In addition, by expression of Flpe recombinase, a conditional knockout allele can be obtained. The sequence and fidelity of *lox*P/*FRT*/*F3* sites is critical for recognition by Cre and Flpe recombinase. The efficacy of *lox*P sites of the targeting constructs was first tested using EL350 which was derived from DY380 (Lee et al., 2001). Besides the λ phage recombination genes found in DY380, EL350 also contained a tightly controlled arabinose-inducible Cre recombinase. Expression of Cre recombinase was induced by addition of arabinose and this facilitated recombination between the two *lox*P sites, resulting in deletion of the intervening sequences. The *Bcl11a-lacZ* and *Bcl11b-lacZ* targeting constructs contained two *lox*P sites. Upon expression of Cre recombinase, the intervening sequence between the *lox*P sites, which contained exon 4 and the *I-Ceu*I restriction digestion site would be deleted (Figure 3.4A). Hence, after Cre recombinase expression, constructs which contained functional *lox*P sites would show a single band (21,529 bp – Bcl11a; 21,807 bp– Bcl11b) following double digestion with *I-Sce*I and *I-Ceu*I. The final targeting constructs were first electroporated into EL350 cells that were either induced or uninduced with arabinose for Cre expression. Cells were then plated into LB agar plates containing Cm. Four Cm^R colonies were picked for each construct and analysed by restriction digestion. As shown in Figure 3.4A, all four clones of each targeting constructs showed a single band after *I-Sce*I and *I-Ceu*I double digestion following Cre recombinase expression. In contrast, constructs that were electroporated into EL350 without Cre recombinase expression retained the *I-Ceu*I site. Therefore restriction digestion with *I-Sce*I and *I-Ceu*I resulted in the presence of two bands, the vector backbone (~21 kb) and the *lacZ-Puro* insert (6,814 bp) (Figure 3.4A). Hence, the *lox*P sites in both *Bcl11a-lacZ* and *Bcl11b-lacZ* constructs were functional.

Next, I verified the fidelity and efficacy of the *FRT*/*F3* sites using EL250. EL250 was also derived from DY380; however, in contrast to EL350, EL250 contained a tightly controlled arabinose-inducible Flpe recombinase gene (Lee et al., 2001). Expression of

Flpe recombinase was induced by addition of arabinose and this would facilitate recombination between the two identical *FRT*/*F3* sites, resulting in removal of the *lacZ-Puro* and *Neo* cassettes from the *Bcl11a-lacZ* and *Bcl11b-lacZ* constructs. The *Bcl11a-lacZ* and *Bcl11b-lacZ* targeting constructs were first electroporated into EL250 cells that were either induced or uninduced with arabinose for Flpe expression. Next, the transformants were plated out onto LB agar plates containing either Kan or Cm. Clones that contained constructs in which the *lacZ-Puro* and *Neo* cassettes were successfully excised would be sensitive to Puro and Kan but resistant to Cm because the Cm^R coding sequence was found on the PL611 plasmid backbone. In contrast, clones which contained constructs with partial/no excision of the *lacZ-Puro* and *Neo* cassettes would remain $Puro^R$ and/or Kan^R. The number of Kan^R colonies observed would reflect the efficiency of Flpe recombination reactions. Flpe recombinase-mediated recombination was fairly efficient as there were fewer than 10 Kan^R colonies. In contrast, there were about 1000 Cm^R colonies. Four Cm^R colonies from each construct were selected and checked for their Kan^R. All four clones from the *Bcl11a-lacZ* construct were susceptible to Kan. In contrast, three of the Cm^R colonies from the *Bcl11b-lacZ* construct showed resistance to Kan (plasmid 2, 3 and 4). Next, these clones were tested using the 5' and 3' primer pairs (Figure 3.4B) to confirm that the *lacZ-Puro* and *Neo* cassettes were properly excised. Constructs with *lacZ-Puro* (5' PCR) and *Neo* (3' PCR) cassettes successfully excised would show a 5' PCR band of 454/471 bp (*Bcl11a*/*Bcl11b*) and a 3' PCR band of 488/532 bp (*Bcl11a*/*Bcl11b*) respectively (Figure 3.4B). All four Cm^R clones of the *Bcl11a-lacZ* construct showed complete excision of *lacZ-Puro* and *Neo* cassettes following Flpe recombinase expression (Figure 3.4B). In contrast, for the *Bcl11b-lacZ* construct, all four clones showed complete *Puro-lacZ* cassette excision but only one showed complete *Neo* cassette excision (Figure 3.4B). This result confirmed the previous observation that only one of the four Cm^R clones was susceptible to Kan (plasmid 1). As the other three clones (plasmids 2, 3 and 4) had incomplete/no excision of the *Neo* cassette, resistance to Kan was retained. This result also suggests that recombination between *F3* sites is not as efficient as *FRT* sites. However, this inefficiency would not pose a problem when using germline Flpe mice as Flpe recombinase would be expressed constitutively. In addition, in agreement with the study by Schlake *et al.* (Schlake and

Bode, 1994), no recombination between *FRT* and *F3* sites were detected, suggesting that recombination occurred only between two *F3* sites or wild-type *FRT* sites.



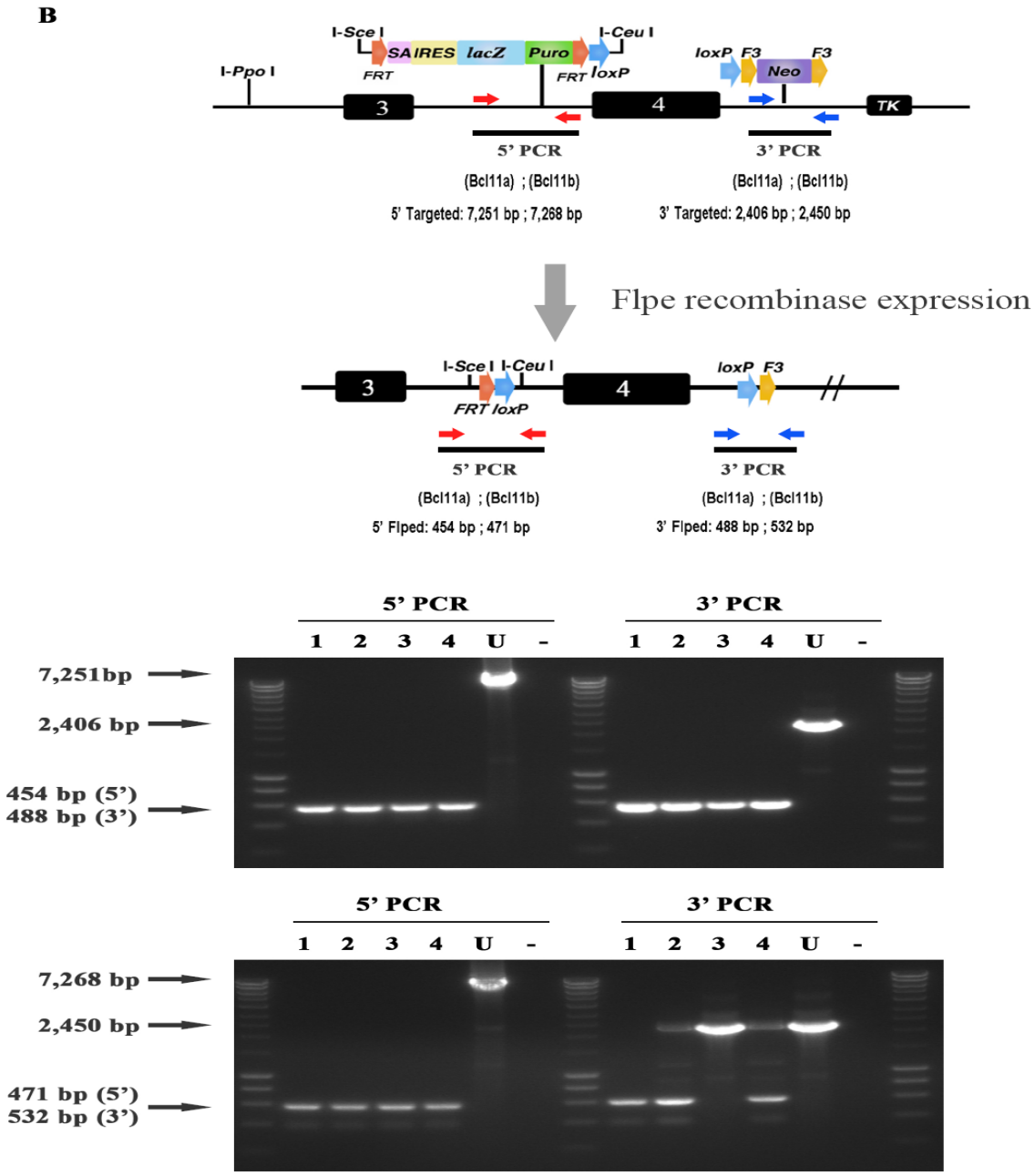
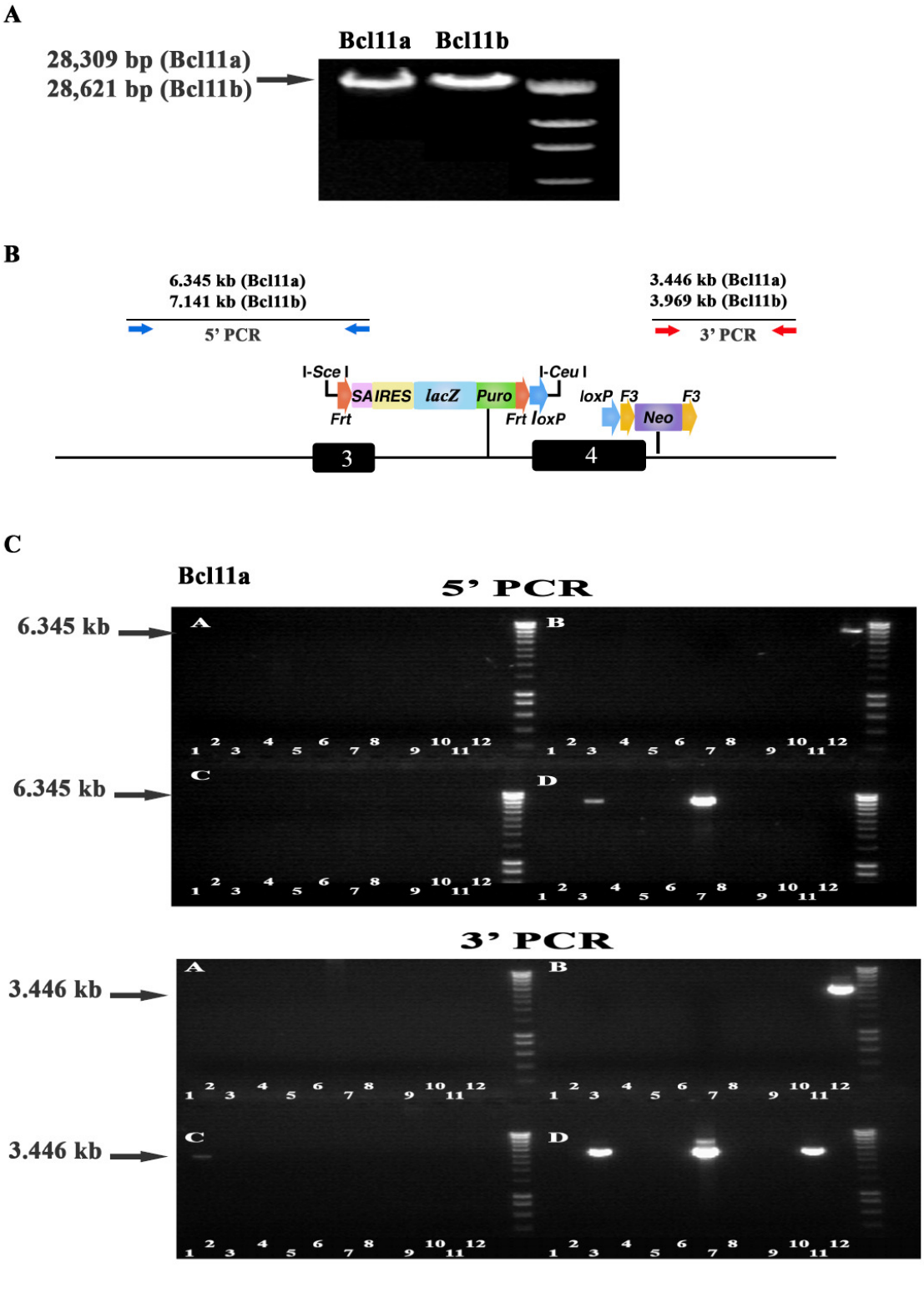


Figure 3.4. Verification of *lox*P/*FRT*/*F3* functionality. (A) Restriction digestion patterns of *Bcl11a* and *Bcl11b* targeting constructs with *I-Sce*I and *I-Ceu*I following Cre recombinase expression. Successful recombination between *lox*P sites results in elimination of *I-Ceu*I enzyme site. Therefore restriction digestion with *I-Sce*I and *I-Ceu*I produces a single band (Lanes 1-4) without the presence of the 6,814 bp *lacZ-Puro* insert as seen in the original constructs (Lane U). **(B)** Gel images showing PCR verification of *Bcl11a* and *Bcl11b* constructs following Flpe recombinase expression. Primer pairs used are as shown. Blue and red arrows indicate location of primers used for 5' and 3' PCR respectively. Successful recombination between *FRT* sites or *F3* sites results in elimination of the *lacZ-Puro* and *Neo* cassettes respectively. Hence PCR amplification of these plasmids (Lanes 1-4) produces a 454 bp product (5' PCR) and a 488 bp product (3' PCR) for *Bcl11a* and a 471 bp product (5' PCR) and a 532 bp product (3' PCR) for *Bcl11b*. Lane U shows the PCR product obtained from original targeting vectors without Flpe recombinase expression.

3.2.3 Targeting to ES cells and verification of targeted clones

The *Bcl11a-lacZ* and *Bcl11b-lacZ* final targeting constructs were subsequently linearized by *I-Ppo*I (Figure 3.5A) and electroporated into AB2.2 wild-type ES cells. Gene targeting events were identified by selecting for cells resistant to G418. DNA from G418-resistant ($G418^R$) colonies were analysed for the desired homologous recombination events with two independent long-range PCR reactions (5' and 3' PCR). Each of these primer pairs consists of a common primer that resides within the *SA* (5' PCR) or *Neo* cassette (3' PCR) and a gene-specific primer that resides outside the homology arms (Figure 3.5B). Correctly targeted ES cell clones would produce a 6,345/7,141 bp (*Bcl11a*/*Bcl11b*) and a 3,446/3,696 bp (*Bcl11a*/*Bcl11b*) band for the 5' and 3' long range PCR reactions respectively. As shown in Figure 3.5C, for *Bcl11a-lacZ* targeting, 3 of the 48 $G418^R$ colonies analysed showed positive long range PCR results for both 5' and 3' reactions, giving an overall targeting efficiency of at least 6.25%. In contrast, for *Bcl11b-lacZ* targeting, 8 of the 48 $G418^R$ colonies analysed showed positive long range PCR results for both 5' and 3' reactions, giving an overall targeting efficiency of at least 16.67% (Figure 3.5D).


A
Bcl11a Bcl11b
28,309 bp (Bcl11a)
28,621 bp (Bcl11b)
B
6.345 kb (Bcl11a)
7.141 kb (Bcl11b)
5' PCR
3.446 kb (Bcl11a)
3.969 kb (Bcl11b)
3' PCR
I-Sce I
SA
IRES
lacZ
Puro
I-Ceu I
loxP
F3
F3
Neo
Frt
Frt
loxP
3
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B
C
D
3' PCR
3.446 kb
3.446 kb

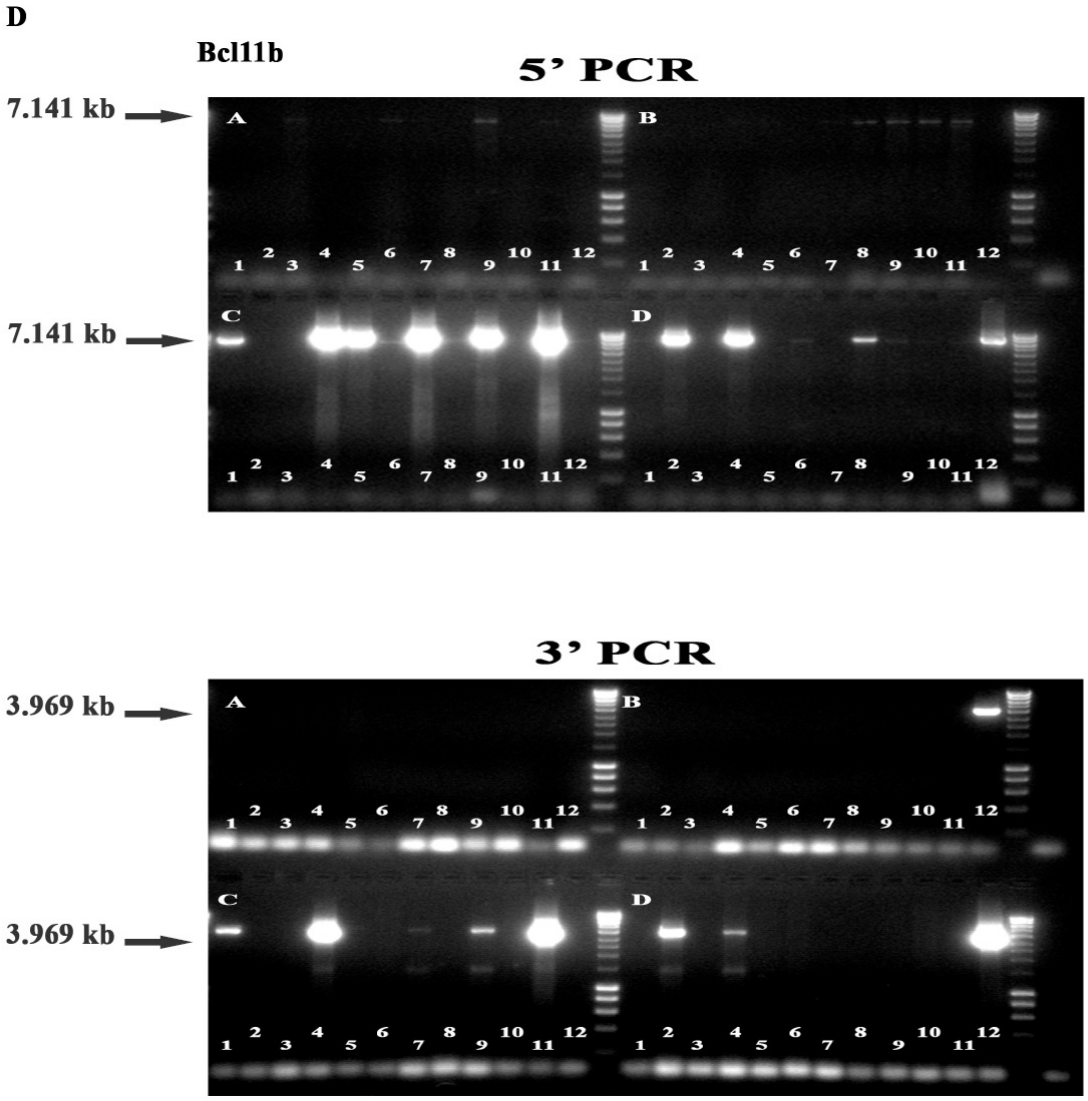

Figure 3.5. ES cell targeting. (A) Gel image showing linearized *Bcl11a* and *Bcl11b* final targeting vectors prior to electroporation into ES cells. **(B)** Schematic diagram showing the *Bcl11-lacZ* reporter alleles. Blue and red arrows indicate location of primers used for long range PCR confirmation of 5' and 3' targeting respectively. Long range PCR verification of **(C)** *Bcl11a-lacZ* and **(D)** *Bcl11b-lacZ* targeted ES clones using 5' and 3' primers. Numbers represent individual colony picked and analysed.

3.2.4 Confirmation of germline transmission of targeted alleles

Two independent targeted clones for each construct were injected into 3.5 dpc C57BL6/J mouse blastocyts which were subsequently implanted into uteri of pseudopregnant foster females (Microinjections were performed by Dr Si Qin and Tina Hamilton). Chimeras were obtained from each microinjection (Table 3.1). F0 chimeric mice are not typically phenotyped directly because they are comprised of a mixture of wild-type and mutant cells. Male chimeras were therefore bred to wild-type C57BL6/J females to generate F1 generation offspring. Genomic DNA was extracted from F1 offspring and genotyped using long-range PCR with primers as described in Figure 3.5B to assay for germline transmission of targeted alleles. As shown in Figure 3.6A, germline transmission of *Bcl11a-lacZ* and *Bcl11b-lacZ* targeted alleles were obtained in F1 mice. These heterozygotes were then intercrossed to produce F2 generation heterozygotes and/or homozygotes which were characterized to assess gene function. Having confirmed germline transmission of the targeted alleles by long-range PCR, mice were subsequently genotyped using two short PCR reactions. The position of primers used were as illustrated in Figure 3.6B; with a pair of gene-specific forward (Fwd) and reverse (Rev2) primers amplifying the wild-type band (*Bcl11a* – 363 bp; *Bcl11b* – 644 bp) and the gene-specific forward (Fwd) primer with a common reverse (Rev1) primer located within *SA* of the *lacZ-Puro* cassette amplifying the mutant band (*Bcl11a* – 437 bp; *Bcl11b* – 527 bp). PCR of genomic DNA from heterozygotes would produce two bands; a wild-type and a mutant band while genomic DNA from wild-type would produce only a wild-type band (Figure 3.6C).

Table 3.1. Chimeras obtained from microinjections

Allele	Clone/Passage number	% chimera
Bcl11a-lacZ	B12/P6	20
Bcl11a-lacZ	B12/P6	40
Bcl11a-lacZ	B12/P6	40
Bcl11a-lacZ	B12/P6	50
Bcl11a-lacZ	D7/P5	50
Bcl11a-lacZ	D7/P5	30
Bcl11b-lacZ	C11/P6	30
Bcl11b-lacZ	C11/P6	10
Bcl11b-lacZ	D2/P5	60
Bcl11b-lacZ	D2/P5	30

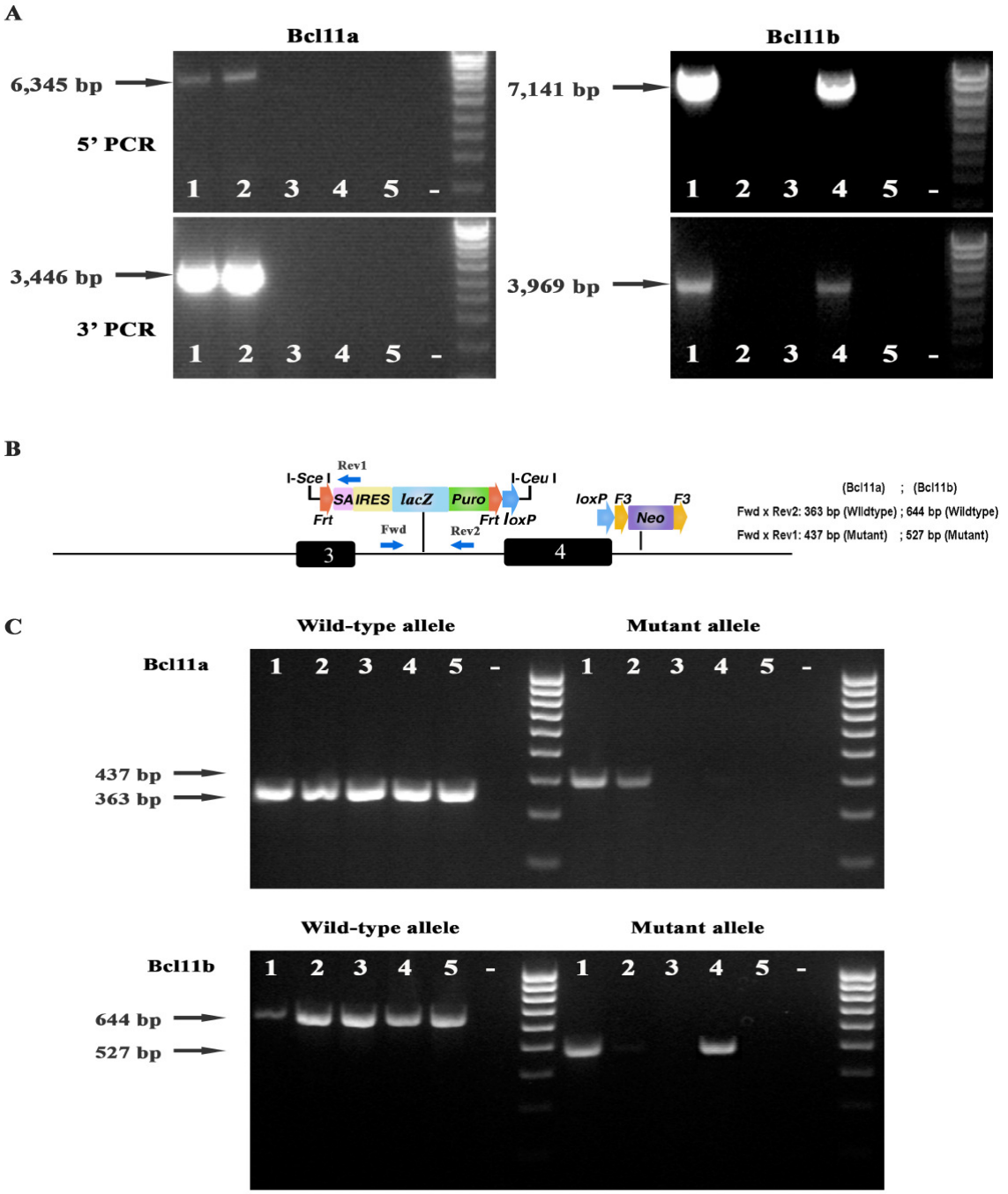

Figure 3.6. Confirmation of germline transmission of targeted alleles in F1 mice. Five F1 mice were characterized for targeted *Bcl11a-lacZ* and *Bcl11b-lacZ* alleles. **(A)** Gel images showing 5' and 3' long range PCR confirmation of germline transmission of targeted alleles in *Bcl11a-lacZ* and *Bcl11b-lacZ* F1 mice using long-range PCR. **(B)** Genotyping using short-range PCR. Blue arrows indicate positions of new genotyping primers. PCR amplification of tail genomic DNA with Fwd and Rev2 primer pairs produces a 363 bp (*Bcl11a*) and a 644 bp (*Bcl11b*) wild-type band. PCR amplification of tail genomic DNA with Fwd and Rev1 primer pair produces a 437 bp (*Bcl11a*) and a 527 bp (*Bcl11b*) mutant band. **(C)** The 363 bp wild-type band is present in all five *Bcl11a-lacZ* mice while the 437 bp mutant band is present only in mice 1 and 2 indicating these two mice are heterozygotes. The 644 bp wild-type band is present in all five *Bcl11b-lacZ* mice while the 527 bp mutant band is present only in mice 1 and 4 indicating these two mice are heterozygotes.

3.3 Discussion

I have used the new mobile recombineering reagents (λ phage and pSim) to generate targeting constructs for *Bcl11a-lacZ* and *Bcl11b-lacZ* reporter conditional null alleles. These two constructs were verified by expression of Cre and Flpe recombinase using EL350 and EL250 and the functionality and fidelity of *lox*P, *FRT* and *F3* sites were confirmed. I then electroporated these targeting constructs to wild-type ES cells and successfully isolated ES cells which contained the targeted alleles. These ES cell clones were confirmed by long range PCR genotyping. Two independent clones for each construct were then microinjected into 3.5 dpc mouse blastocyts and transplanted into uteri of pseudopregnant females. The chimeras obtained were then bred to wild-type females and the resultant F1 mice born were genotyped by long range PCR to confirm germline transmission of the targeted alleles. Heterozygous *Bcl11a-lacZ* and *Bcl11b-lacZ* mice were obtained.

During verification of the functionality and fidelity of the constructs, I observed that the recombination efficiency between *F3* sites was not as efficient as that of *FRT* sites. Three out of the four *Bcl11b-lacZ* clones picked showed partial recombination between *F3* sites (Figure 3.4B). However, it should be noted that expression of Flpe recombinase was only induced for one hour (in this experiment) and this inefficiency could be negated by increasing the duration of expression of Flpe recombinase. Moreover, this inefficiency should not pose a problem *in vivo* because Flpe recombinase is driven by the *Rosa26* promoter; hence Flpe recombinase is expressed constitutively and complete recombination between *F3* sites is expected and indeed achieved.

In Chapter 4, I will detail the expression patterns of *Bcl11* genes using the *Bcl11-lacZ* reporter mice. I will characterize the spatial expression patterns using whole mount X-gal staining and also delineate the expression patterns at a single cell level using flow cytometric analysis.