CHAPTER 4. ATTEMPT TO GENERATE A *Blm*-DEFICIENT CELL LINE WITH BAC ACCEPTOR

4.1 Introduction

Homozygous deletions can occur in a *Blm*-deficient mutation library, followed by irradiation and the loss of heterozygosity (LOH). To complement mutant gene function, integrating BAC genomic DNA into a specific locus is a possible approach. BACs are able to carry original genomic sequences and cis-regulatory elements of a gene. Thus, the expression of the introduced gene is more likely expressed in its original status. In addition, single copy transgenes through BACs avoid unpredicted effects resulted from various copy numbers. In this chapter, the strategy to generate a *Blm*-deficient ES cell line with a BAC acceptor at the *Hprt* locus is described.

The gene-targeting technique was used to knockout the *Blm* gene. Through homologous recombination, exons 2 and 3 of the *Blm* gene were replaced by a positive and negative selection cassette *PGK/EM7/neo-MC1-tk* which was flanked by *FRT* sites. This cassette can be selected in *E. coli* by Kanamycin through expression of the *Neo* cassette driven by the *EM7* promoter; it can also be selected by G418 through the expression of the *Neo* cassette driven by the *PGK* promoter. The whole selection marker can be removed by Flpe through the flanking *FRT* sites. Loss of *MC1-tk* can be selected with FIAU. The deletion of exon 2 and 3 of the *Blm* gene disrupts the downstream reading frame and results in loss-of-function of the BLM protein.

The strategy pursued in this project is to generate homozygous mutations with large deletions which can be identified by array comparative genomic hybridization (CGH). However, there will not be enough evidence to conclude which single gene is associated with the phenotype given that deletions range from several hundred kilo base pairs to million base pairs. Recovering the normal phenotype by expression of candidate gene facilitates the identification of the causal mutation. When a normal gene, introduced from outside of a cell, converts a mutant phenotype to a normal phenotype, it can be concluded that the gene is responsible for the mutant phenotype. There are several ways to introduce a gene into cells including: I. Integrating cDNA into a genome to achieve stable expression. II. Introducing the genomic locus, for example by integrating bacterial artificial chromosomes (BACs) into a genome to achieve stable expression.

A cDNA can complement a mutant's function by providing the correct transcripts. Using this principle, a technology called expression cloning been used in many systems, for instance to clone the co-receptor of human immunodeficiency virus (Deng *et al.* 1997) and the co-receptor of hepatitis C virus (Evans *et al.* 2007). In these projects, cDNA libraries were transformed into a specific cell line, which should not be infectable by the type of virus. Cells which gain the ability to be infected after cDNA transformation can be used to identify the specific cDNA, which codes for the protein which restores viral infection. The drawback of expression cloning is that some genes have very long transcripts therefore cDNAs of these genes are under represented in cDNA libraries, which limits the applicability of this method. In addition, some other reasons limit the application of the expression cloning, for instance, not all cDNAs express appropriately in cell lines; and alternative splice structures can be lost through cDNA expression; and physiological expression levels are hard to achieve.

BACs have insertions with lengths ranging from 100-200 kb of genomic DNA thus are long enough to host most genes with their regulatory elements. Thus, introduction of BACs into a cell can restore gene function when they are integrated into the host genome. As a variety of BAC libraries are ready to use, introducing a BAC into a cells is a convenient approach to restore the normal function of mutant genes.

Cells with a BAC acceptor locus can efficiently integrate large DNA fragments into the genome to provide stable gene expressions. This kind of integration can be achieved either randomly or locus specifically. Random integration is easier to do and saves time. But it can result in multiple copies of transformed genes (Antoch *et al.* 1997) and cannot ensure intact DNA integration (Chandler *et al.* 2007). Also insertion sites vary thus gene expression levels are much more variable (Korn *et al.* 1992; Nobrega *et al.* 2003). Locus specific integration can be achieved by recombination mediated cassette exchange (RMCE) (Baer *et al.* 2001), which generates a single copy integration of the exogenous DNA. RMCE requires a recombinase such as Cre. A genomic DNA fragment flanked by two hetero-specific *lox* sites can be replaced by a plasmid or BAC DNA fragment flanked by the same two *lox* sites. In this way, the expression of the integrated gene can be well controlled.

The murine X-linked *Hprt* locus is a relatively neutral site for RMCE since mutations in this locus do not impair mouse viability (Kuehn *et al.* 1987; Kushi *et al.* 1998). In addition, it has been shown that BACs can be integrated into the mouse *Hprt* locus and transmitted through germ line transmition. Mice with such a BAC expresses in the expected tissue specific

manner (Heaney *et al.* 2004). I planned to modify an existing BAC acceptor cell line CCI18 (Prosser 2007 unpublished). This cell line is wild type for *Blm* function thus it is necessary to generate a *Blm*-deficient cell line with the ability to integrate genomic DNA fragments from a BAC into the *Hprt* locus. In the CCI18 cell line, a *loxP*- and *Lox511*- flanked *PuroΔtk* cassette was targeted to intron 2 of the *Hprt* gene through homologous recombination. Although this is inserted in the gene the function of *Hprt* is not disrupted.

As I needed to use the *PuroΔtk* cassette in the following retroviral infection experiments, I replaced the *PuroΔtk* cassette in the CCI18 cell line with a blastistidin selection cassette (*Bsd*) through RMCE. Then the *Blm* gene was targeted by a targeting vector with a *FRT* sites flanked *PGK/EM7/neo-MC1-tk* cassette. By recycling the selection marker through Flpe recombinase, both alleles of *Blm* gene can be mutated.

The Mouse BAC libraries RPCI-23/24 have genomic DNA fragments flanked by the *loxP* and *Lox511* sites. After introducing a eukaryotic selection marker, for example the *neomycin* gene between two *lox* sites, mouse genomic DNA in these BAC libraries can be easily introduced into *Hprt* locus by Cre mediated RMCE. A single copy of genomic DNA can be integrated at the *Hprt* locus, resulting in a loss-of-function of *Hprt*. RMCE cells can be isolated by G418 for integration of *neomycin* gene and by 6TG for the inactivation of the *Hprt* gene. Regulated by the integrated gene's own regulatory elements, functional expression will be achieved.

4.2 Results

4.2.1 Generation of the ZK6 wild type BAC acceptor cell line

The ZK6 *Bsd* BAC acceptor cell line was generated by replacing *PuroΔtk* in the CCI18 cell line through recombination mediated cassette exchange between the cassette provided by pZK33 and the *PuroΔtk* cassette at the *Hprt* locus (Figure 4-1). Bcyd selection results in two types of cells: correct RMCE events and the random integration of the *Pcmv-EM7-BSD-SV40pA* cassette. As a control the *Bsd* RMCE plasmids pZK33.4 and pZK33.3 were transformed into 1–3×10^7 CCI18 cells separately without CRE. After electroporation the cells were selected in Bcyd and FIAU. Five and one colony grew in the above plates, respectively, which mean background is low.

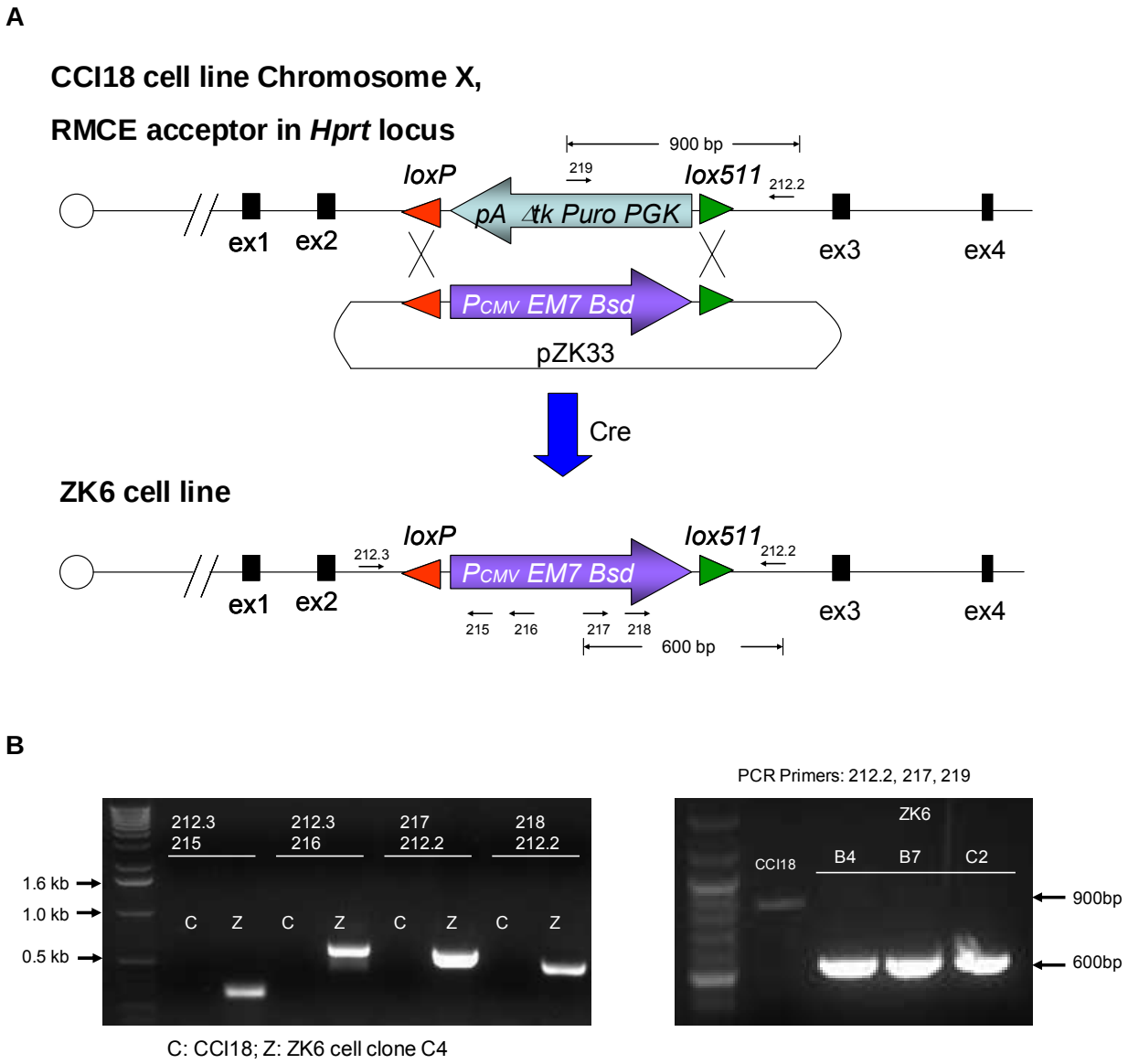

Figure 4-1 Generation of ZK6 BAC acceptor cell line

A. Schematic of RMCE event and position of genotyping primers. CCI18 contains the *PuroΔtk* cassette while ZK6 contains the *CMV-EM7-Bsd* cassette. Ex: exon; **B**. PCR results using the primer pairs 212.3/215, 212.3/216, 212.2/217, 212.2/218 confirmed ZK6 clone C4 has the correct junction fragment compared with CCI18 cells. PCR results using primers 212.2, 217 and 219 confirmed the genotype of CCI18 and ZK6 clones B4, B7 and C2.

To replace the *PuroΔtk* cassette by RMCE, a Cre expression plasmid pCAG-Cre was transformed into CCI18 cells by electroporation. The *loxP-Bsd-lox511* cassette was then introduced with Cre expression plasmids by electroporation in two experiments between one and five days later. These time courses were used to explore optional conditions for a high percentage of RMCE events. Each electroporation was split into two plates for selection in Bcyd and FIAU or Bcyd only. Colonies growing in Bcyd and FIAU media will represent those with the designed RMCE events plus random insertions in cells, which have lost *tk* expression. Colonies growing in Bcyd media only will be those with RMCE events plus those with a random insertion of the *Bsd* cassette.

The results of this assessment are illustrated in Table 4-1. After 5 days of the first introduction of the Cre recombinase, a higher RMCE frequency could be achieved. In the first experiment, cells were electroporated with pCAG-Cre one day before the RMCE construct and the pCAG-Cre (the 2nd time) were introduced. In this case, 10% of the Bcyd resistant clones were RMCE events. In the cells transfected with pCAG-Cre 5 days before the RMCE construct and the pCAG-Cre (the 2nd time) were introduced, 100% of the Bcyd resistant clones were RMCE events.

As designed, this RMCE introduces a *Bsd* resistant gene and removes the *PuroΔtk* gene, therefore Bcyd and FIAU double resistant clones should have the desired genetic change. These clones were confirmed by PCR genotyping. Primers were designed as shown in Figure 4-1. All primers were tested and confirmed that they can specifically amplify DNA fragments using CCI18 cells or pZK33 *Bsd* RMCE plasmid as templates in the preliminary experiments (data not shown). When RMCE does not occur, primer 212.2 in *Hprt* and the primer 219 in the *PuroΔtk* cassette can amplify a 900 bp fragment by PCR (Figure 4-2). After correct RMCE, the PCR product generated with 212.2 and 219 in the CCI18 cells should not be generated. Endogenous *Hprt* primers 212.2 and 212.3 can amplify several junction DNA fragments with the primers 215, 216, 217 and 218, which are in the *Pcmv-EM7-Bsd-SV40pA* cassette. This was confirmed using CCI18 and the clone C4 of ZK6 cell (Figure 4-1).

68 Bcyd and FIAU double resistant clones were genotyped by PCR. Sixty five of them had a 600 bp PCR product from correct RMCE using primers 212.2, 217 and 219 (Figure 4-2). When using CCI18 *PuroΔtk* BAC acceptor cells as the template, this 600 bp junction PCR product was not produced. These 65 double resistant clones can be regarded as different clones of ZK6 cell line, named as ZK6: A1–A4, A6–A12, B1–B12, C1–C5, C7–C12, D1–D2, D4–D12, E1–E12, F1–F8.

Table 4-1 The efficiency of RMCE

Time	Cell number per transformation	$Bcyd^R$ colony	$Bcyd^R$+$FIAU^R$ colony	$(Bcyd+FIAU)^R/Bcyd^R$
1 day	1.2×10^7	180	18	10%
5 day	3.0×10^7	41	68	~100%

The different sets of plasmids were transfected into CCI18 cells. Then cells were grown in normal media for 1 or 5 days before applying the drug for selection. 2-3 days additional Bcyd selection was used to maintain experiment time course comparable. These data indicated cells with transformed Cre plasmid 5 days before the introduction of RMCE cassette had increased RMCE percentage.

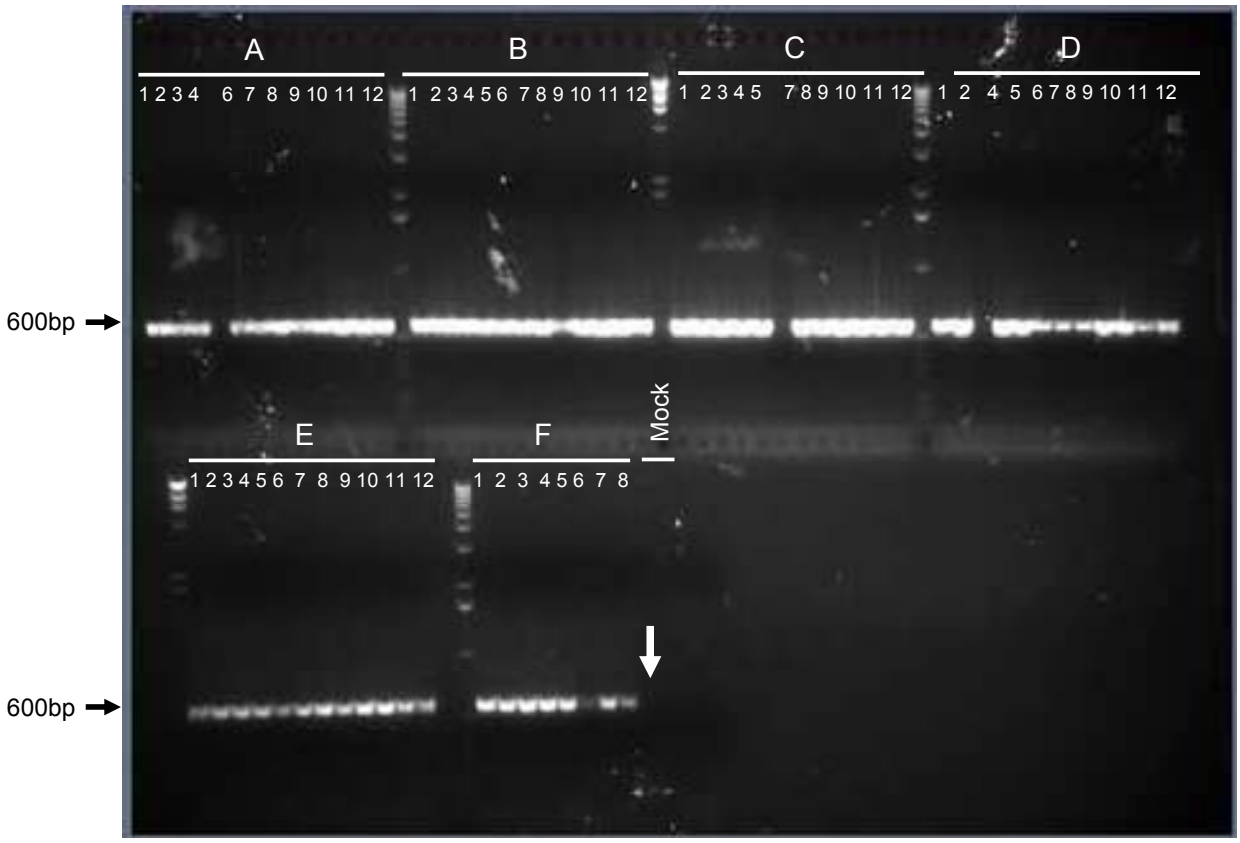

Figure 4-2 Genotype of all ZK6 BAC acceptor clones

Sixty eight Bcyd and FIAU double resistant clones were genotyped by a PCR strategy using primers 212.2, 217 and 219. Sixty five ZK6 clones, except the clones A5, C6 and D3, appeared positive by PCR genotyping with a 600 bp band according to primer design in Figure 4-1. CCI18 (mock) cell should have a 900 bp band (white arrow), which however is very weak here. This band can be seen in Figure 4-1 B.

4.2.2 Confirmation that RMCE can be achieved

BACs from library RPCI-23, 24 (http://bacpac.chori.org/home.htm) are suitable resources to complement normal function of mutant mouse genes. Prior to RMCE, these BACs need to be modified by recombineering to carry a *Neo*mycin/*Kanamycin* marker for both prokaryotic and eukaryotic selection. The *loxP* and *Lox511* sites on the backbone pBACe3.6 (Frengen *et al.* 1999) enable the large genomic DNA fragments to be introduced into the X-linked *Hprt* locus of ZK6 by RMCE (Figure 4-3). The suitability of my cell line ZK6 for RMCE was confirmed by PhD student Qi Liang who introduced fragments of the *p53* gene into the *Hprt* locus of ZK6. This RMCE event was selected in G418 first for 5 days for the integration of the *neomycin* cassette. Then cells were selected in 6TG containing media because 3 exons of *p53* interrupt the transcript of the *Hprt* gene which results in 6TG resistance. $6TG^R$ cells were confirmed to have gained the *p53* gene fragment and removed the P_{CMV}-*EM7-Bsd* cassette.

4.2.3 *Blm*$^{+/-}$ BAC acceptor cell lines

To generate a *Blm*-deficient BAC acceptor cell line with ability to incorporate BAC fragments through RMCE, the *Blm* gene-targeting vector was electroporated into the ZK6 cell line. After G418 selection clones were picked into 96-well plates. Half of the volume from each well was expanded and the other half was used to genotype the targeting event by PCR (Figure 4-4).

PCR primers were tested before being used for genotyping. A total of ~1000 $G418^R$ clones were generated in 3 electroporations. Two hundred eighty-eight clones were picked and screen by PCR. Three were positive. One of them was subcloned and confirmed by Southern blot analysis (Figure 4-4).

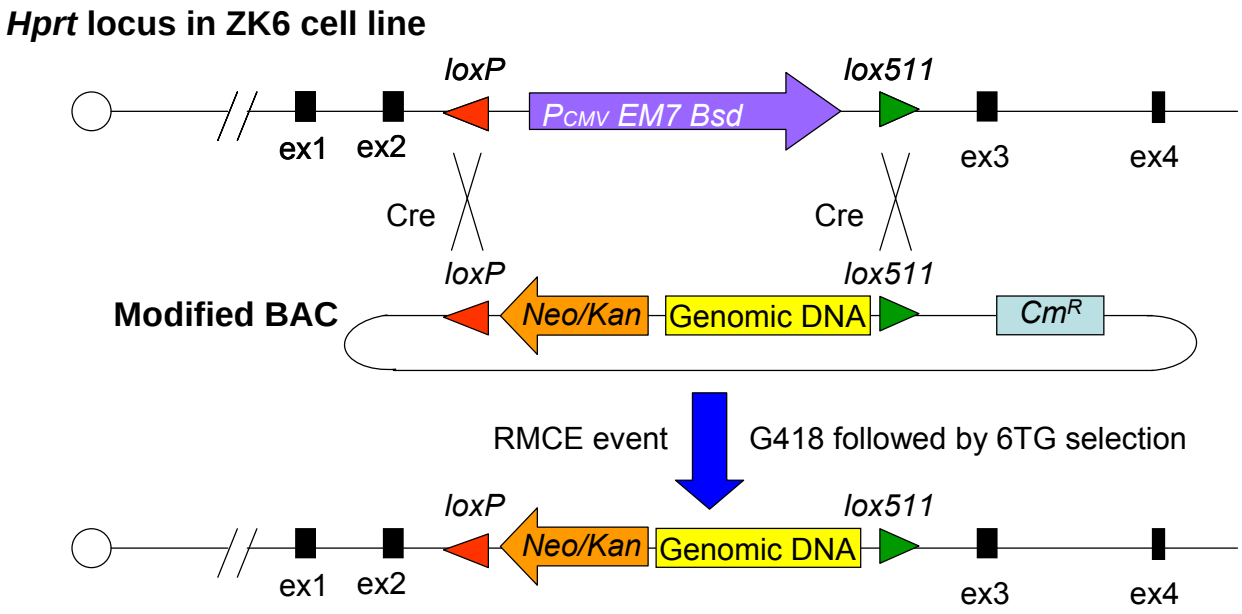

Figure 4-3 The principle of RMCE in ZK6 cell line

Recombination between *Lox* sites is catalysed by Cre. As a result of RMCE, genomic DNA (inserted a *Neo*mycin/*Kanamycin* cassette) is introduced into the *Hprt* locus of ZK6. RMCE events can be selected in G418 and 6TG, for the introduction of the *Neomycin* cassette and loss-of-function of *Hprt* function, respectively. These two selective chemicals can be used sequentially (G418 first) to avoid interfering with cell viability. Ex: Exon. *Neo*/*Kan*: *Neo*mycin/*Kanamycin* cassette.

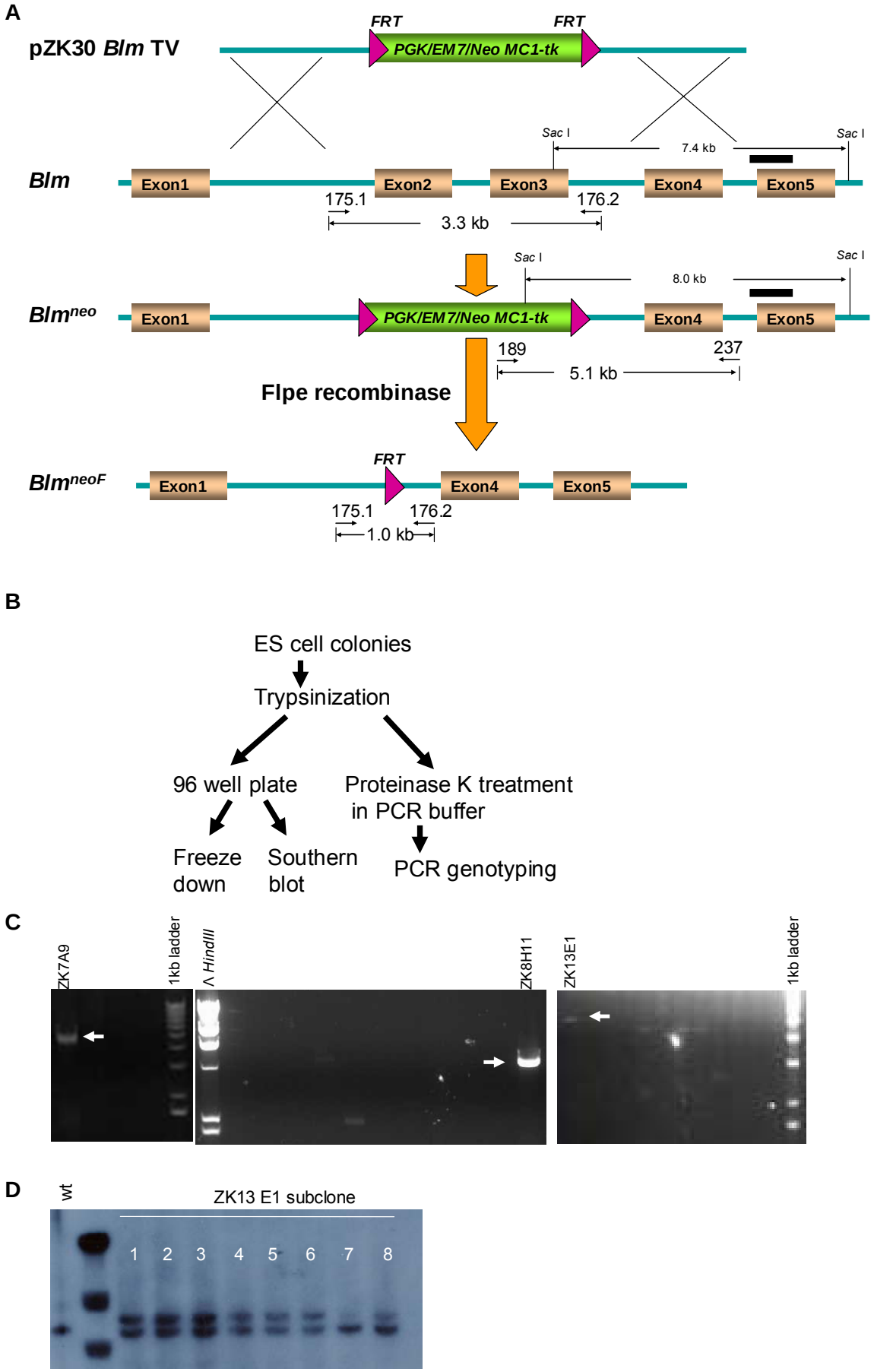

Figure 4-4 ***Blm*** **gene-targeting strategy**

Figure 4-4 *Blm* gene-targeting strategy

A. Schematic view of the *Blm* gene-targeting vector (pZK30 *Blm*TV), wild type allele (*Blm*), targeted allele (Blm^{neo}) and neomycin pop-out allele (Blm^{neoF}). pZK30 *Blm* gene-targeting vector contained the selection cassette *PGK*/*EM7*/*neo-MC1-tk*, which was flanked by *FRT* sties. Exon 2 and 3 in the wild type *Blm* allele were replaced to generate the Blm^{neo} allele. After Flpe recombinase mediated recombination, the selection marker could be popped out, leaving a *FRT* site at the allele Blm^{neoF}. Primers 189, 237, 175.1 and 176.2 were designed to genotype cells. *Sac* I digestion was used to distinguish wild type and targeted alleles by Southern blot. Black bar was probe 185. **B**. Experiment design after colonies were picked. When colonies were trypsinized, a half volume of cells were harvested and lysed. Crude DNA served as a template in PCR genotyping. The other half volume of cells was expanded in 96-well plates and passaged for DNA extraction and subsequent Southern blot analysis. **C**. PCR genotyping showed that clones ZK7A9, ZK8H11 and ZK13E1 had the correct products. PCR primers 189 and 237 amplify a 5.1 kb band from the targeted allele. **D**. Southern blot analysis confirmed that ZK13 E1 subclones 1–6 were $Blm^{+/-}$ BAC acceptor cells. Wild type cells showed one 7.4 kb band, while targeted alleles showed an 8.0 kb band. The probe was zk185 (the black bar in a.).

4.2.4 Using Flpe/*FRT* to remove the selection marker

The selection marker had to be popped out from *Blm*$^{+/-}$ cells by Flpe to reuse the same targeting vector for targeting the second allele of the *Blm* gene. Before doing this, the targeted clones ZK7A9, ZK8H11 and ZK13E1 needed to be subcloned as 1–10% cells in the original colonies may not be targeted and thus will contribute significantly to the background. 5' and 3' PCR genotyping confirmed that the single cell subclones ZK7A9.1and ZK8H11.2 had correct alleles (Figure 4-5). To test the *MC1-tk* function, ZK7A9.1 and ZK8H11.2 were plated in FIAU (Figure 4-6). Both clones appeared to be FIAU-resistant. Therefore, second round of gene-targeting was carried out to isolate a new *Blm*$^{+/-}$ clone which resulted in clone ZK13E1. This was single cell subcloned as before and Southern analysis confirmed that all the subclones were *Blm*$^{+/-}$ (Figure 4-4), but these clones were also FIAU-resistant. The plasmids used in these transformations were subcloned and then transformed into CCI18 cells to test whether their selection markers were functional. The result indicated that they all contained functional *neomycin* and *HSV-tk* gene (Figure 4-7). This proved that the selection cassettes in the targeting vector were functional. Thus, the parental cells of the heterozygously targeted *Blm*$^{+/-}$ cells may contain a pre-existing mutation in the FIAU metabolism pathway, which blocks the killing effect of FIAU. This phenomenon is similar in that in Chapter 3.

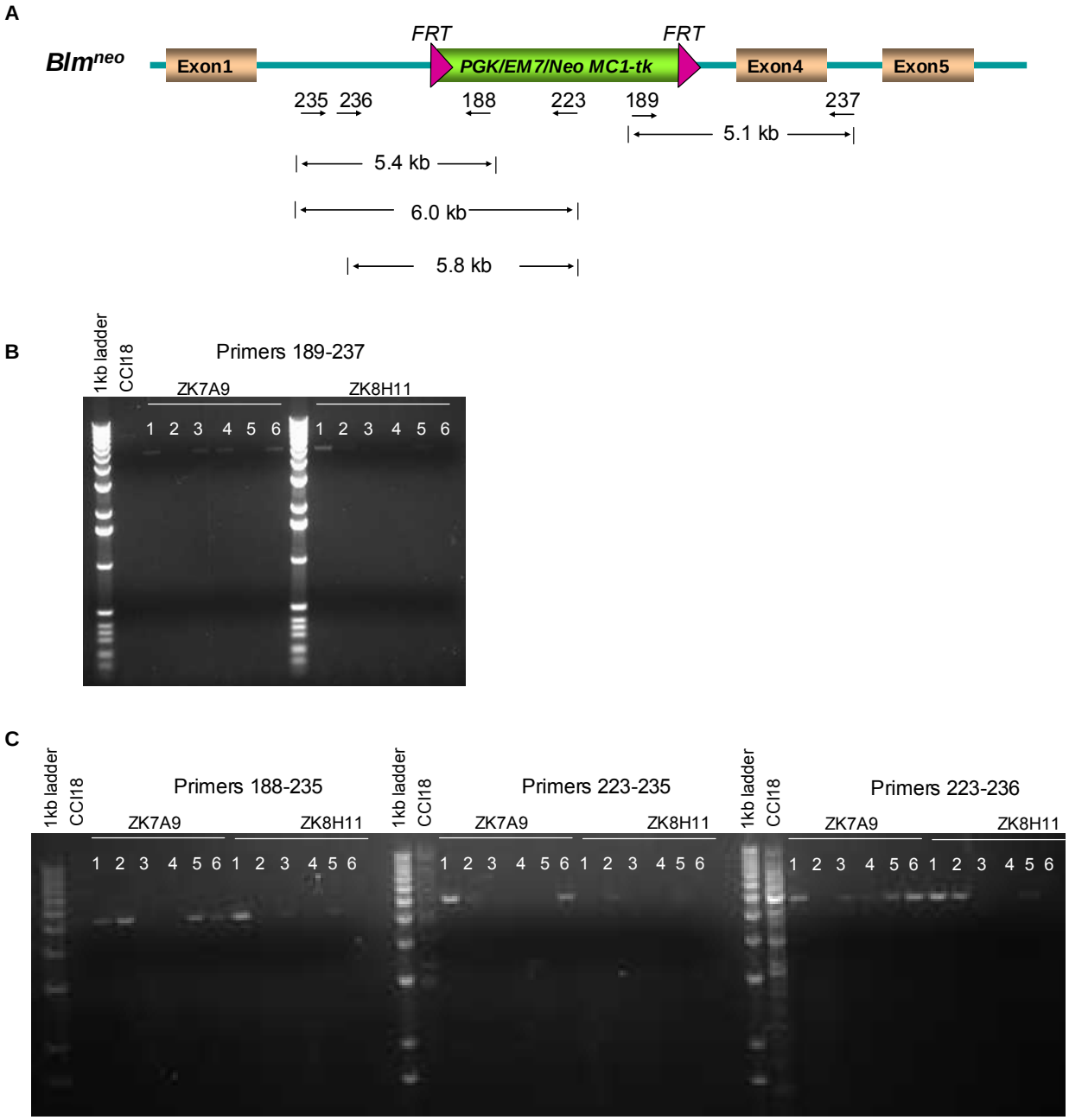

Figure 4-5 PCR genotype of ZK7A9 ZK8H11 subclones

A. Schematic view of primer design to PCR genotype pZK30 targeted *Blm*^{neo} allele. **B**. 3' PCR genotype. Primer pair: zk189-237. Expect a 5.1 kb product. **C**. 5' PCR genotype. Primer pairs: zk188-235, zk223-235 and zk223-236. Expected products were 5.4 kb, 6.0 kb and 5.8 kb in length, respectively.

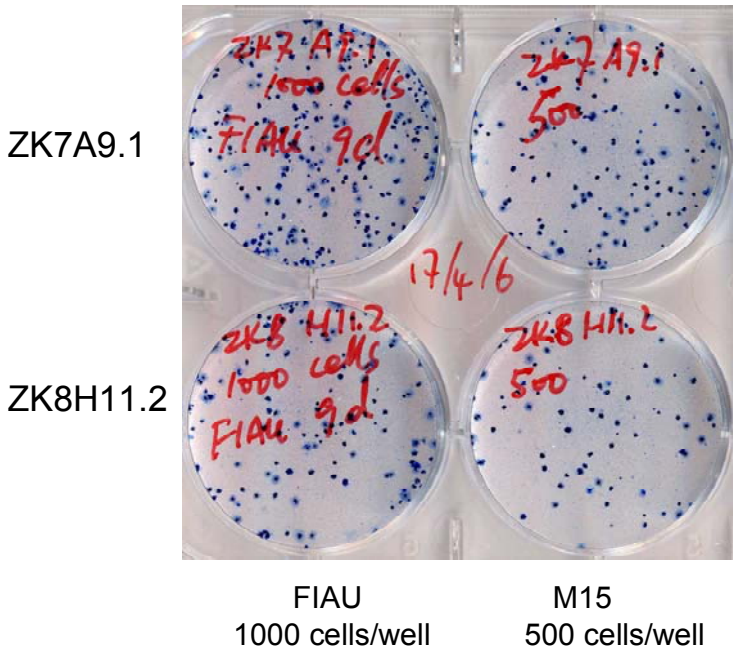

Figure 4-6 FIAU-resistant background of ZK7 and ZK8 cells

Two subclones of *Blm* heterozygous targeted cells ZK7A9.1 and ZK8H11.2 were cultured in M15 normal media (500 cells) and FIAU (1000 cells) in 30 mm wells. Cells were stained by methylene blue after nine days culture. Both clones are resistant to FIAU.

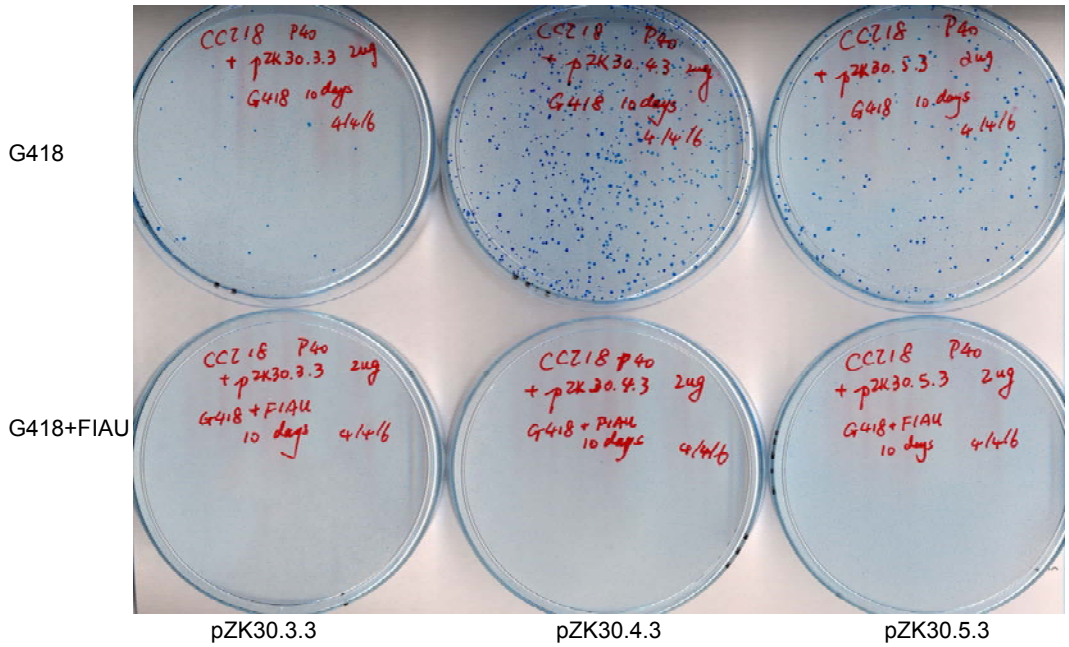

Figure 4-7 Selection confirmed functional markers in *BIm* targeting vectors

Not I linearized plasmid pZK30.3.3, 30.4.3 and 30.5.3 (4ug each) were transformed into CCI18 cells. Each electroporation was split into two plates evenly for either G418 selection or G418+FIAU selection in the following 10 days. Cells were stained by Methylene Blue (2%). Three electroporations produced G418 resistant colonies but not G418+FIAU-resistant colonies, which indicated that 3 types of plasmids had functional *Neomycin* and *HSV-tk* genes.

4.2.5 Generation of the *Blm*^{neoF} allele

Since the isolated *Blm*$^{+/-}$ clones ZK7A9, ZK8H11 and ZK13E1 were resistant to FIAU, it was impossible to select for FIAU^{R} clones resulting from the loss of the marker following Flpe mediated recombination. However, considering the efficiency of Flpe-mediated recombination is around 1%, the clones that have ocurred Flpe-mediated recombiantion can still be achieved and isolated without the FIAU selection.

The clonally purified derivatives of the targeted clones ZK13E1.1, ZK13E1.2 and ZK13E1.3 were electroporated with the Flpe expression plasmid pCAG-Flpe, resulting in ZK15, ZK16 and ZK17, respectively. A PCR genotyping strategy (Figure 4-4, A) using the primers zk175.1 and zk176.2 confirmed that clones ZK15 C11, F10, G1 and ZK16 H2 had the desired popped out *Blm*^{neoF} allele (Figure 4-8). Although PCR using these two primers could also generate a 3.3 kb wild type fragment, the short extension time of PCR did not allow this to occur.

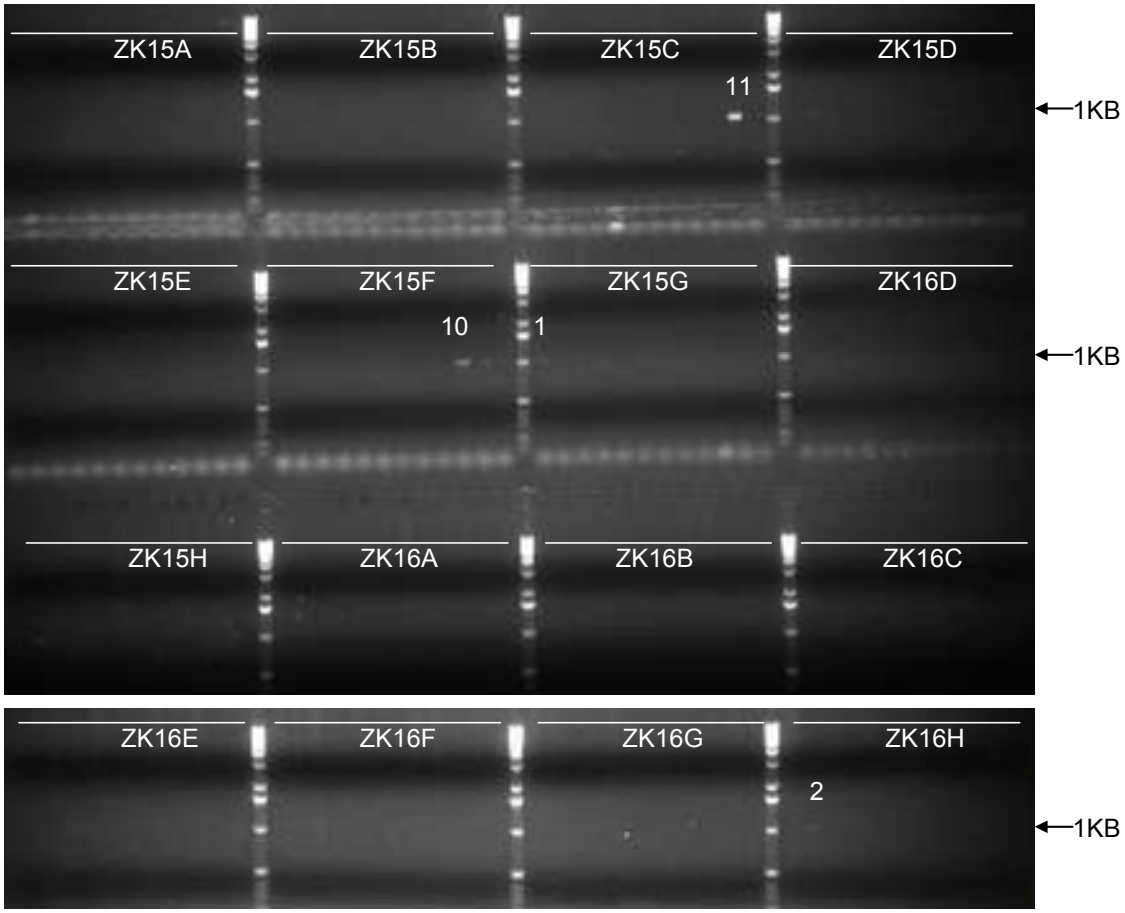

Figure 4-8 PCR screen of ZK15 and ZK16 clones

1kb PCR products were expected by amplifying DNA with primers zk175.1 and zk176.2. Clone ZK15 C11, F10, G1 and ZK16 H2 contained a 1kb band.

4.3 Discussion

A *Blm*-deficient ES cell line with a BAC acceptor locus would be a valuable resource to study many biological pathways, which are active in mouse ES cells. Following the successful screens done with *Blm*-deficient ES cells (Guo *et al.* 2004; Wang and Bradley 2007), more loss-of-function screens can be designed and implemented using this system. To generate a *Blm*-deficient ES cell line with a BAC acceptor, an existing wild type BAC acceptor cell line (CCI18) was used. CCI18 has a *loxP* and *lox5-11* flanked *PuroΔtk* cassette, which was replaced by a *loxP* and *lox5-11* flanked *Bsd* cassette through recombination mediated cassette exchange (RMCE). The two experiments within different time frames showed that the RMCE efficiency can vary from 10% to 100% depending on the time point of introducing RMCE cassette with more Cre (Table 4-1). When the RMCE construct and the Cre plasmid were electroporated 5 days later than the first introduction of the Cre recombinase, 100% $Bcyd^R$ and $FIAU^R$ cells were correct RMCE events. This increased efficiency of RMCE may be achieved by the accumulated Cre protein in the cells.

By switching the markers from *PuroΔtk* to *Bsd*, a MMuLV recombinant virus containing the *PuroΔtk* cassette can be used to infect cells and to generate molecular tags in the future. A *Blm* gene-targeting vector (pZK30) was constructed to delete the exon 2 and 3 of the gene. With a *PGK/EM7/neo-MC1-tk* cassette flanked by *FRT* sites which can be selected in *E. coli* and eukaryotic cells and recycled using Flpe mediated recombination. Although the *PGK/EM7/neo-MC1-tk* cassette was shown fully functional when random integrated into the wild type cells (Figure 4-7), the targeted clones appeared to have lost the function of *HSV-tk*. This inactivation of the *HSV-tk* cassette may be due to the genome instability caused by *Blm* heterozygosity. It has been known that chromosomal breakage in human spermatozoa was increased in two out of three fathers (Mutant *BLM* carriers) of persons with Bloom syndrome (Martin *et al.* 1994). Moreover, the carrier frequency (1.8%, 23/1244) of the mutant BLM^{Ash} allele in colorectal cancer samples is higher than that (0.8%, 85/10,099) in historical controls from healthy people (Gruber *et al.* 2002). And mice heterozygous for the *Blm* gene demonstrated the *Blm* haploinsufficiency developed twice the number of intestinal tumours when crossed with the mice carrying a mutation of the *Apc* tumour suppressor gene (Goss *et al.* 2002). The dis-function of the *HSV-tk* can be resulted from small mutations causing reading frame shifts. The mechanism of the spontaneous inactivation of the *HSV-tk* cassette may be similar or associated to that of the *Δtk* cassette in the *Blm* deficient background, described in Chapter 3.

When Flpe was used to remove and recycle the *PGK/EM7/neo-MC1-tk* cassette, FIAU selection could not be used to isolate cells which had excised the *PGK/EM7/neo-MC1-tk* cassette. A PCR strategy was used to screen a large number of clones to identify those with the popped out allele. These two cells are heterozygous for the *Blm* gene and ready to be targeted again to generate a homozygous *Blm* mutant cell line with a BAC acceptor.