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Chapter title: Establishment of the DNA repair-defective mutants in DT40 cells

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Summary

The chicken B cell line DT40 has been widely used as a model system for reverse genetics studies in higher eukaryotes, because of its advantages including efficient gene targeting and ease of chromosome manipulation. Although the genetic approach using the RNA interference technique has become the standard method particularly in human cells, DT40 still remains a powerful tool to investigate the regulation and function of genes and proteins in a vertebrate system, because of feasibility of easy, rapid, and clear genetic experiments. The use of DT40 cells for DNA repair research has several advantages. In addition to canonical assays for DNA repair, such as measurement of the sensitivities toward DNA damage reagents, it is possible to measure homologous recombination and translesion synthesis activities using activation-induced deaminase (AID)-induced diversification of the immunoglobulin locus. In this chapter, we would describe a detailed protocol for gene disruption experiments in DT40 cells.

1. Introduction

The chicken DT40 B cell line was established from Avian Leukosis Virus-induced lymphoma in the Bursa of Fabricius (1). DT40 cells display the high ratio of targeted to random DNA integration, which occurs at essentially all loci with efficiencies that are orders of magnitude higher than those observed in mammalian cells (2). Targeted integration of DNA constructs by homologous recombination (HR) enables us to inactivate a gene of interest (knock-out). Alternatively, we can modify a specific aspect of a gene function by the introduction of a more subtle mutation (knock-in). These approaches are essentially the same as those in the yeast *S. cerevisiae*, or in the murine embryonic stem (ES) cells to produce mutant mouse strain.

Recently, genetic approaches using RNA interference techniques (“knock-down” experiments) have been widely used for higher eukaryotes including *C. elegans*, *Drosophila*, and human cells. However, it always remains a possibility that the reduction in the target mRNA/protein expression level might be insufficient or the off-target effects may occur. DT40 knockout cell lines are much easier to make compared to mouse ES cells. Therefore, gene targeting experiments in DT40 cells still remain a powerful option to investigate the regulation and function of gene and protein in vertebrate cells. This is particularly true if the species (chicken) and cell types (lymphocytes) are not a major concern.

DT40 has a number of advantages as a model system. First of all, the cells grow very rapidly and therefore its handling is easy. Second, the gene targeting procedure has been established very well. We will describe our protocol in this chapter. Third, a number of mutant cell lines have already been created and they constitute a useful panel. Forth, highly sophisticated experiments are possible such as creation of double, conditional, or knock-in mutants. Fifth, DT40 cell has a relatively invariant character in both karyotype and phenotype even during extended period of cell culture. Therefore, data from DT40 cells could be directly compared mutants by mutants. In contrast, cells from human patients or knock-out mice from different genetic backgrounds are more difficult to compare. Sixth, DT40 cell is also a good source for biochemical analyses, because of the ease of large-scale culture with the stable characters under the same genetic background.

The use of DT40 cells for DNA repair research has some advantages. In

addition to canonical assays of DNA repair, such as measurements of the sensitivities to DNA damage reagents, several unique DNA repair assays are available. First, the homologous recombination (HR) and translesion synthesis (TLS) activities can be measured using the intrinsic property of DT40 that diversifies its immunoglobulin (Ig) antigen specificity (3). This is depending on expression of activation-induced deaminase (AID), transcription of the Ig gene, and HR and TLS mechanisms (3)- (4). The efficiency can be measured by simple subculture and FACS analysis of the reexpression of the surface Ig. Second, the gene targeting efficiency can be measured by transfection of a targeting construct (2). Targeting and non-targeting events can be determined by Southern blotting or PCR analysis. Third, the repair efficiency of the chromosomal double strand break (DSB) in an artificially integrated recombination substrate at a specific locus can be measured (5). DSB is introduced by plasmid-based expression of the rare cutting enzyme I-SceI (6).

As in other systems, there are a few drawbacks in DT40. Since they are chicken cells, availability of the reagents can be a problem. In particular, antibodies against human or mouse proteins often do not cross-react with the chicken counterparts. Knock-in of an epitope tag including a green fluorescent protein (GFP) could overcome this problem. Another potential problem is that chicken genome sequence data are far from complete, even though the draft of chicken genome had published in 2004 (7). This may cause a trouble in designing targeting vector or comparative genomic hybridization array. In addition, there might be some chance of missing information in the database if you attempt to identify proteins by mass spectrometry. Finally, we have to take into account the absence of functional p53 in DT40, which induces apoptosis and cell cycle arrest upon DNA damage.

2. Materials

2.1 Maintenance of DT40 cell line. Culture medium is RPMI 1640 with L-glutamate, supplemented with 10% fetal calf serum (FCS), 1% chicken serum, 2 mM L-glutamate, and 50 μ M β -mercaptoethanol. Addition of penicillin and streptomycin is optional. FCS is pre-heated at 56°C for 30 min to prevent complement activation. Chicken serum need not be heat-inactivated.

2.2 Gene pulser II Electroporator (Biorad).

2.3 Antibiotics used for selection of DT40 transfectants (Table 1)

2.4 Standard set up for molecular biology lab: Reagents for plasmid construction, PCR, and Southern blotting.

3. Methods

3.1 DT40 cell culture.

Optimal growth conditions for DT40 cells are in complete RPMI 1640 medium (see Materials 2.1) at 39.5°C with 5% CO₂. The doubling time is about 8 hr in this condition (Notes 1). For long-term storage, cells suspended in FCS containing 10% DMSO could be frozen at -80°C or in liquid nitrogen. For short-term storage of clones awaiting Southern blotting, we mix one volume of cultured cells with the same volume of FCS containing 20% DMSO in an 1.5 ml tube and put them directly into deep freezer.

3.2 How to design a gene targeting construct

You should consider the following points.

1. How to obtain chicken DNA sequences. Prior to design a gene targeting vector, you need to identify the chicken cDNA and genomic sequences of the gene of your interest in the database, for example, at National Center for Biotechnology Information (NCBI) Web site (<http://www.ncbi.nlm.nih.gov/>). See Notes 2 and 3.
2. Components of the vector. Gene targeting constructs contain a selection drug maker cassette flanked by genomic sequences at both sides (often called left and right or upstream and downstream arms), and a vector backbone. Any cloning vector can be used for this purpose. In DT40 cells, the gene targeting efficiency is very high, thus you do not need negative selection.
3. How you would like to disrupt the gene of your interest? By gene targeting event, the selection gene cassette is inserted into or replace the gene of interest. The size of the deleted genome is determined by the position of left and right arms within the genome. To fully inactivate a gene, it is theoretically best to replace the

complete coding sequences with the cassette. However, this is not always easily achievable because a large distance between the arms may drastically decrease the ratio of targeted to random integration, though more than 20 kb of the gene locus has been deleted (8). As a compromise, we usually design a vector with the deletion size not exceeding 10 kb, and try to delete exon(s) encoding a critical domain of the protein in interest. We also place the boundary of the arm within an exon to ensure disruption of the coding sequence. Introducing an in-frame stop codon might help.

4. The length of the arms. This would greatly affect the efficiency of gene targeting, and generally speaking the longer the better. However, compared with mouse ES cells system that requires more than 10 kb of the genomic sequences, shorter genomic sequences have been used in DT40 cells: 2-5 kb arms usually resulted in successful targeting. Our minimal arm length that worked was 0.8 kb (9). We usually design our constructs in such a way that the size of individual left and right arm of are more than 1.5 kb, the combined size of the arms is more than 3 kb (preferably 4-5 kb) and less than 10 kb.
5. All of our selection drug marker cassette are flanked by *Bam*HI sites and can be conventionally cloned into *Bam*HI (or *Bgl*II) sites located between the left and right arms. It is best to use *Bam*HI, since it is sometimes necessary to swap the cassette with another one. A number of the selection marker gene cassettes are available upon request (Table). At the moment we have *hisD*, *bsr*, *puro* cassettes flanked with loxP sequences, and they are useful for selection marker recycling to make double or triple knockout. Our first choice is the *hisD* and *bsr* cassettes, since the drugs kill cells rapidly, and are not expensive. Puromycin selection is also very efficient, however, we tend to save it for re-expression experiments.
6. The targeting vector should normally be linearized before transfection, and it is best to consider which enzyme site to use while designing the vector. We often use *Pvu*I or *Sca*I that are located in the *bla* (ampicillin resistant) gene of the vector backbone, or a restriction enzyme, which cut a unique site in the multicloning sites flanking the either arm. The linearization seems to increase the number of the transfectants obtained after the selection.
7. Screening strategy. We normally screen transfected clones for targeting events with Southern blotting. You should consider the enzyme appropriate for screening and

DNA fragment used for probe. The probe should not hybridize with the arms. An alternative screening method is described in Notes 4.

8. DT40 is diploid except for chromosome 2, which is in trisomy. If the gene of your interest is on chromosome 2, unfortunately you will have to delete three alleles with three serial transfections with three different selection drugs. On the other hand, since DT40 was derived from a female chicken, and it has only one chromosome Z (chicken sex chromosome). Human chromosome 9 and chicken chromosome Z are in synteny (10), thus a gene in human chromosome 9 is expected to be deleted in a single gene targeting event in DT40 (11) - (12). See Notes 5.

3.4 Construction of the gene targeting vector

We describe an example for the construction using pBluescript vector.

1. Select the restriction sites for cloning both arms, which do not present in the arm sequences. In the example shown in the Figure 1, we use for *SalI*-*Bam*HI sites for left arm and *Bam*HI-*NotI* sites for right arm.
2. Synthesize PCR primers incorporating the restriction sites at the ends (Figure 1A).
3. Amplify the target arms by long-range PCR (e.g., Takara LA-Taq) using designed primers and genomic DNA from DT40 cells as template. This will produce arm sequence isogenic to at least one allele of DT40 cells and would enhance the targeting efficiency. LA-Taq may create a number of mutations in the arms, but this seems not to affect targeting efficiency.
4. Cloning the left arm into *XhoI*-*Bam*HI sites and right arm into *Bam*HI-*SpeI* sites of pBluescript (Figure 1B). Confirm the arms by sequencing, for example, using M13 Forward and/or Reverse primers, and by restriction digestion.
5. Finally, the drug marker cassette is inserted into *Bam*HI site (Figure 1C). Check the orientation of the drug marker cassette by restriction enzyme digestion and/or sequencing. We prefer the drug resistance cassette placed in reverse orientation to the direction of transcription of the gene. It seems good practice to save the vector with another orientation as well, since sometimes the vector with one orientation shows better targeting efficiency compared to the vector with the other.

3.5 Transfection in DT40. The DT40 cells are readily transfected with DNA by electroporation.

1. Linearize 30 μ g plasmid DNA (targeting vector) with a restriction enzyme in sufficient volume of buffer (e.g., 200 μ l and 60-90 units of enzyme), then precipitate with ethanol and Sodium Acetate and dry up. Extraction of DNA with phenol/chloroform is not essential.
2. Spin down $0.5-1 \times 10^7$ cells. Use cells in log phase. Aspirate medium.
3. Resuspend cells in 0.5 ml PBS and transfer to a cuvette with 4 mm gap (Biorad or BTX).
4. Dissolve linearized DNA in 20 μ l of PBS, and add to the cuvette. Mix well. Avoid forming bubbles.
5. Incubate on ice for 10 min.
6. Electroporate with Gene pulser II (Biorad) at the condition of 0.55 kV and 25 μ F. Record time constant each time to verify conditions.
7. Incubate on ice for 10 min.
8. Transfer cells to 20 ml fresh medium in 10 cm dish. Culture cells for 16 to 24 hours.
9. Spin down cells in two conical 50 ml tubes (~10 ml cells / each tube).
10. Resuspend cells in ~80 ml fresh medium (~40 ml /each tube). Add appropriate amount of selection drugs. Mix well. Plate out into four 96-well plates using an 8-channel pipetter. Put 200 μ l per well.
11. In 5 to 7 days, you will see colonies growing at the bottom of the plates. Usually you will get 20 -100 colonies from one transfection. Pick up colonies by pipette and transfer them to 1 ml medium in 24 well plates when they are less than 2 mm in diameter.
12. After 48 h, expand to ~3 ml culture scale and analyse with genomic Southern blotting to identify clones with correct targeting event.
13. Identified heterozygous targeted clone is transfected again using a vector with different selection drug cassette to disrupt remaining wild type allele. During this second selection process, we normally include two selection drugs to reduce the chance that the second targeting vector might replace the integrated first one.

3.6 Design for conditional gene knockout

Mutants homozygous for genes essential for cell survival or proliferation cannot be produced by the above standard targeted gene disruption. After targeting the first allele by standard methods, further manipulations are required to express the gene product (by a “rescue construct”) that can be shut-off conditionally. Then the second allele should be disrupted. For this purpose, several methods have been used successfully to render DT40 cells conditionally null for essential genes. Each of these methods requires a specific set of plasmids. For details, please see the references indicated.

1. Tet-off system. A conditionally repressible gene is introduced through random integration and the “transgene” is expected to keep the null cell alive by expressing a rescue construct. This rescue construct can be inactivated using the tetracycline repressible promoter (*13*).
2. Cre-loxP system. The Cre recombinase-loxP-mediated deletion system can be used to turn off expression (*14*). An expression cassette might be placed between two loxP sequences in the rescue construct. Alternatively, the loxP sequences can be knocked-in into the gene of interest at appropriate positions flanking important exons. In this case, the expression is driven by the endogenous promoter, which should provide better expression control. The expression is shut off by Cre enzyme fused with the ligand-binding domain of estrogen receptor (MerCreMer) (*15*) upon activation of tamoxifen addition. The efficiency of the Cre-mediated excision was surprisingly high (close to 100%), making this option attractive.
3. A temperature sensitive mutant transgene. This is a possibility since it was used once for CENP-C deletion (*16*).
4. Degron system. Recently, two protein knockdown approaches to rapidly deplete the protein from a rescue construct have been reported. Both methods use a domain to induce degradation (i.e. degron), which is fused to the protein of interest. One is the application of temperature-sensitive degron system using a degradation pathway based on the N-end rule, which has been widely used in yeast (*17*). Another is the auxin-inducible degron system (*18*). Indol-3-acetic acid (IAA), a plant hormone auxin (AUX) directly induce rapid degradation of the AUX/IAA transcription repressors by a specific form of the SCF (Skp1, Cullin and F-box) E3 ubiquitin ligase complexes. Other eukaryotes including vertebrate cells lack the auxin response but share the ubiquitin-based SCF degradation pathway. AUX/IAA bind to F-box

protein TIR1, promote the interaction between SCF-TIR and AUX/IAA transcription repressors, and induce polyubiquitin-proteasome based degradation of the repressor. The auxin-inducible degron system can be introduced by the heterologous expression of TIR1 in non-plant cells, and this application has successfully used in DT40 cells (18).

Notes

1. Lower temperature, for example, at 37°C, is also suitable for DT40 cell maintenance perhaps with some decrease in growth rate. We normally pass the wild-type DT40 cells at every other day at 39.5°C to avoid over growth. The overgrown cells tend to die quickly by apoptosis.

2. Detailed information related to chicken (*Gallus gallus*) genome is obtained at NCBI chicken genome resource page (<http://www.ncbi.nlm.nih.gov/projects/genome/guide/chicken/>). Please keep in mind that the information in the databases are not from DT40, and often contain some differences due to polymorphisms. Do not trust the database too much.

3. If you cannot find any chicken cDNA sequence information in the database, even not in the EST collections, it is worth trying cross hybridization (i.e. Southern blotting of chicken genomic DNA with human or mouse probe) at reduced stringency (19). When you obtain signals, go on to screening the cDNA and/or genomic libraries with that condition.

4. Genomic PCR based screening is also reported (20). In this case, at least one primer should be located outside of the region used in the gene targeting vector.

5. We have encountered once that four transfections were required for disruption of *cbl* gene locus (21).

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Table1 Reagent used for the selection of transfected DT40 cells

Reagent	Supplier	Code	Stock solution	Final concentration
Blasticidin S Hydrochloride	Funakoshi	KK-400	10 mg/ml H ₂ O	25 µg/ml
L-Histidinol dihydrochloride	SIGMA	H6647-5G	50 mg/ml H ₂ O	1 mg/ml
Puromycin dihydrochloride	SIGMA	P8833-25MG	1 mg/ml H ₂ O	0.5 µg/ml
Zeocin	InvivoGen	ant-zn-1	100 mg/ml ^a	1 mg/ml ^b
Hygromycin B	Invitrogen	10687-010	50 mg/ml ^a	2.5 mg/ml ^c
G418 disulfate	nacalai tesque	16513-26	50 mg/ml ^a	2 mg/ml

^aThese are supplied as solution.

^bIn DNA double strand break repair mutant cells, use 1/2-1/4 concentration.

^cAdd 1M HEPES 1 ml per 40 ml culture to neutralize.

Figure 1. Construction of the targeting vector.

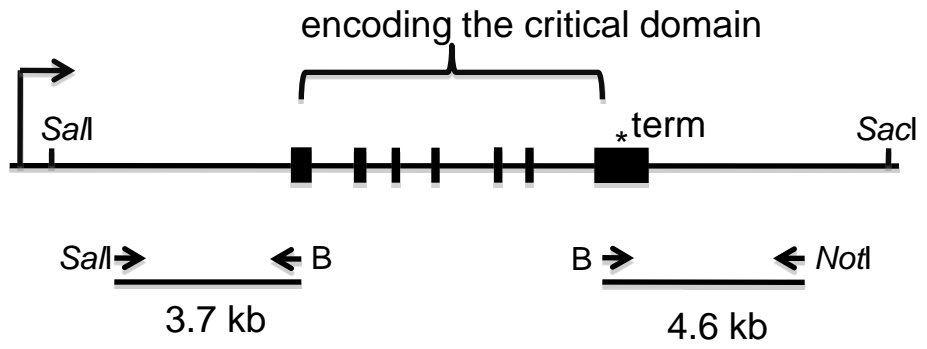
(A) Schematic representation of part of the “gene of interest” allele. For simplification, only one allele is shown. Primers were designed according to DNA sequence of the gene with proper restriction enzyme sites. The fragments of the target vector is amplified by PCR. Black boxes indicate the positions of exons. *term, termination codon; B, *Bam*HI site. (B) Prepared target arm fragments, a drug marker cassette, and the vector (pBluescript) digested with proper restriction enzyme. They are ligated in sequential steps. Triangles indicate loxP sequences. (C) The targeting vector. See details in the text.

Figure 2. Gene targeting of the “gene of interest” loci.

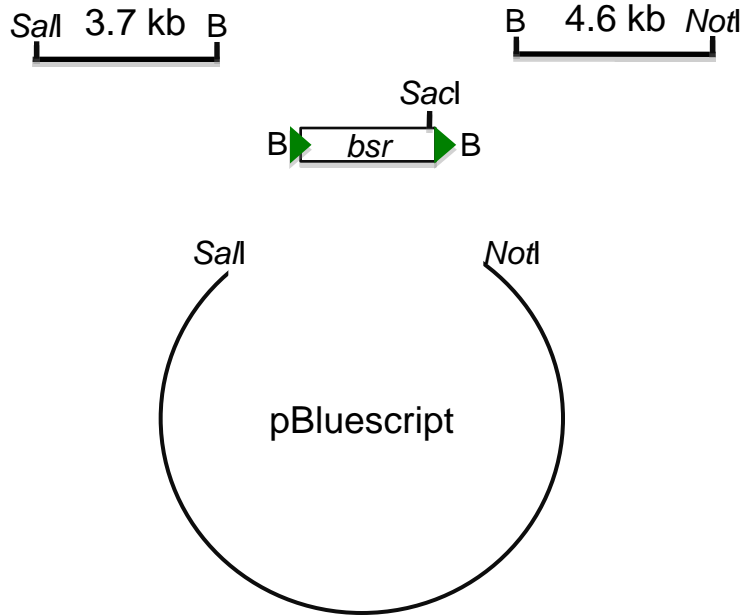
(A) Schematic representation of the “gene of interest”, the gene targeting construct, and the configuration of targeted allele are shown.

(B) Schematic results of Southern blot analysis. In this case, genomic DNA from cells with indicated genotypes are digested with *Sac*I, and probed with the flanking DNA fragment shown as blue box.

A



B



C

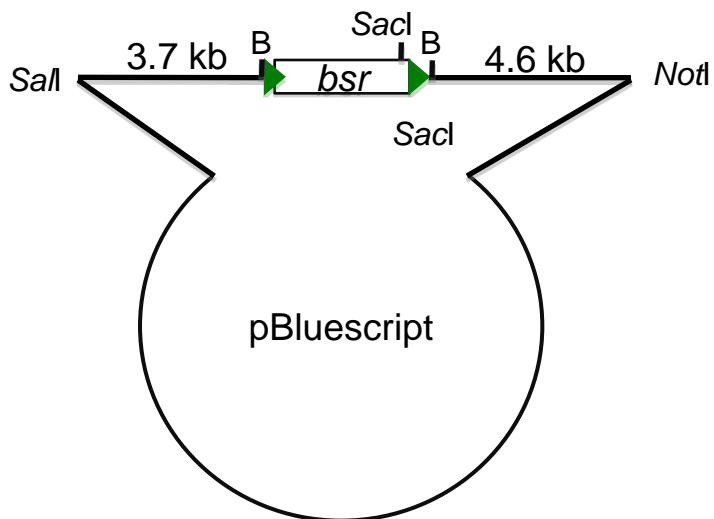


Fig.1

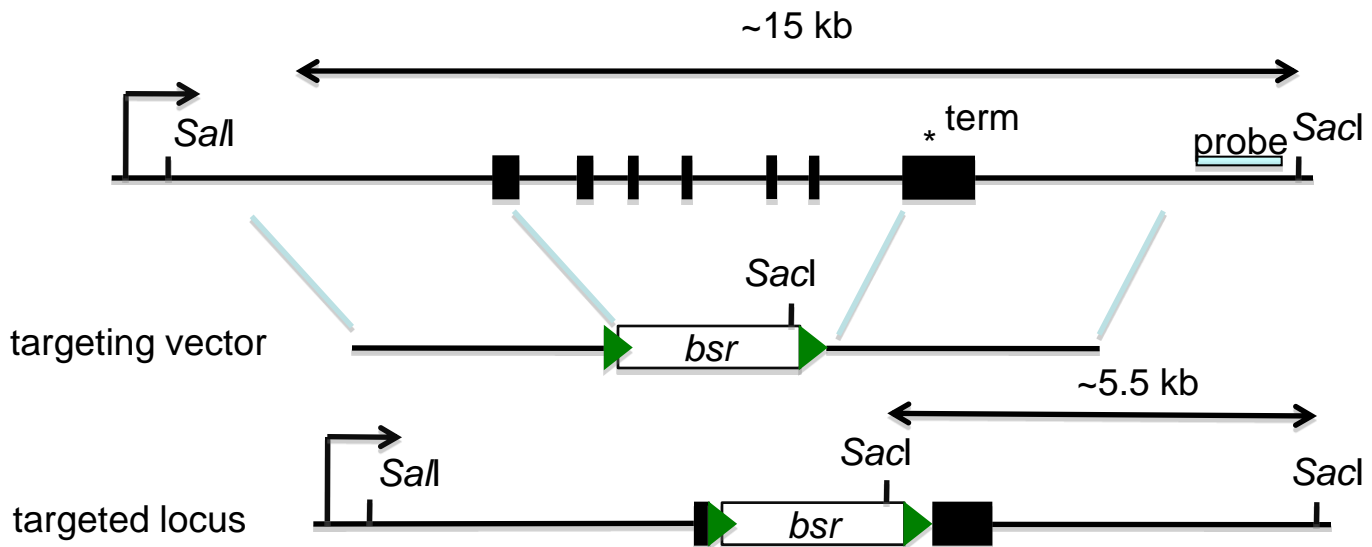
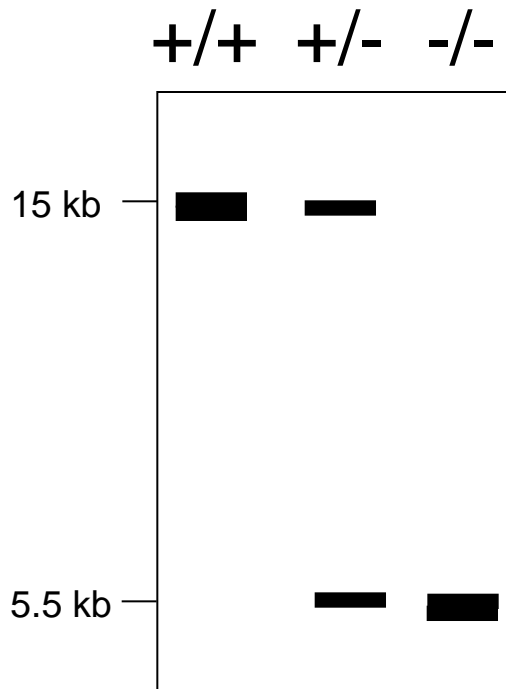
A**B**

Fig.2