Video Article

Identification of *Sleeping Beauty* Transposon Insertions in Solid Tumors using Linker-mediated PCR

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Abstract

Genomic, proteomic, transcriptomic, and epigenomic analyses of human tumors indicate that there are thousands of anomalies within each cancer genome compared to matched normal tissue. Based on these analyses it is evident that there are many undiscovered genetic drivers of cancer¹. Unfortunately these drivers are hidden within a much larger number of passenger anomalies in the genome that do not directly contribute to tumor formation. Another aspect of the cancer genome is that there is considerable genetic heterogeneity within similar tumor types. Each tumor can harbor different mutations that provide a selective advantage for tumor formation². Performing an unbiased forward genetic screen in mice provides the tools to generate tumors and analyze their genetic composition, while reducing the background of passenger mutations. The *Sleeping Beauty* (SB) transposon system is one such method³. The SB system utilizes mobile vectors (transposons) that can be inserted throughout the genome by the transposase enzyme. Mutations are limited to a specific cell type through the use of a conditional transposase allele that is activated by *Cre Recombinase*. Many mouse lines exist that express *Cre Recombinase* in specific tissues. By crossing one of these lines to the conditional transposase allele (e.g. Lox-stop-Lox-SB11), the SB system is activated only in cells that express *Cre Recombinase*. The *Cre Recombinase* will excise a stop cassette that blocks expression of the transposase allele, thereby activating transposon mutagenesis within the designated cell type. An SB screen is initiated by breeding three strains of transgenic mice so that the experimental mice carry a conditional transposase allele, a concatamer of transposons, and a tissue-specific *Cre Recombinase* allele. These mice are allowed to age until tumors form and they become moribund. The mice are then necropsied and genomic DNA is isolated from the tumors. Next, the genomic DNA is subjected to linker-mediated-PCR (LM-PCR) that results in amplification of genomic loci containing an SB transposon. LM-PCR performed on a single tumor will result in hundreds of distinct amplicons representing the hundreds of genomic loci containing transposon insertions in a single tumor⁴. The transposon insertions in all tumors are analyzed and common insertion sites (CISs) are identified using an appropriate statistical method⁵. Genes within the CIS are highly likely to be oncogenes or tumor suppressor genes, and are considered candidate cancer genes. The advantages of using the SB system to identify candidate cancer genes are: 1) the transposon can easily be located in the genome because its sequence is known, 2) transposition can be directed to almost any cell type and 3) the transposon is capable of introducing both gain- and loss-of-function mutations⁶. The following protocol describes how to devise and execute a forward genetic screen using the SB transposon system to identify candidate cancer genes (**Figure 1**).

Video Link

The video component of this article can be found at <http://www.jove.com/video/50156/>

Protocol

1. Breeding and Aging of Transgenic Animals

1. Select the strains of transgenic mice appropriate for your experiment. A typical experiment will use three transgenic lines; a conditional transposase mouse, a mouse harboring a concatamer of transposons, and a mouse expressing *Cre Recombinase* in the cells believed to be the cells of origin for the desired cancer. If the cancer being modeled has a known mutation in a large percentage of the patients it may be desirable to include this mutation at the start. Examples of these "predisposing" mutations include activated *Kras*, dominant negative *Tp53* or a floxed *Pten* allele. By adding in a predisposing mutation the model may better represent the human cancer (see 1.3). Several *Sleeping Beauty* transposase and transposon mice are available from the National Cancer Institute Mouse Repository (<http://mouse.ncicrf.gov/>).
2. Breed conditional transposase mice with mice harboring the transposons to eventually obtain mice homozygous for both alleles if possible. Breed homozygous offspring to mice expressing *Cre Recombinase* to generate your triple transgenic experimental mice (**Figure 2**). Note: Obtaining homozygous mice are not always possible or ideal. For example, homozygous p53 dominant negative mice are much more difficult

to breed than heterozygous mice. In addition, it is helpful to make sure that litters follow Mendelian genetics to confirm that the breeding scheme is successful.

3. If using a predisposed background, first breed mice with the transposase to mice with the predisposing allele. Simultaneously, breed mice expressing *Cre Recombinase* to mice carrying the transposons. Create homozygous mice for each allele if possible. Finally, breed the homozygous offspring from the previous two breeding schemes to generate quadruple transgenic experimental animals.
4. In addition to the experimental animals, generate control cohorts of mice. The control group normally consists of littermates from the experimental breeding that only inherit two of the three alleles (**Figure 2**). In the case of a predisposed background, mice including three out of the four alleles will also be necessary.
5. Monitor experimental and control mice daily for tumor development and/or moribundity. Follow your institutional animal care and use committee (IACUC) requirements for animal husbandry. Also, it is typical to determine an age in which you will sacrifice an animal if it has not yet become moribund. Eighteen months is a typical age at which mice are euthanized.

2. Necropsy of Transgenic Animals

Name of the reagent	Company	Catalogue number
Sure-Seal Induction Chamber	Brain Tree Scientific	EZ-177
Cryovial	Bioexpress	C-3355-2
10% Buffered Formalin	Sigma Aldrich	HT501128-4L

Table 1. Reagents required.

1. When a mouse is moribund or has reached the end of the determined life span, euthanize animal using a CO₂ chamber following your IACUC guidelines. Generally, place the animal in a clean gas chamber, open the gas tank and adjust the regulator to read no higher than 5 pounds per square inch. Fill slowly to minimize nasal and ocular irritation and aversion to CO₂. Make sure that the animal's heart is no longer beating and does not respond when touched in the eye.
2. Spray exterior of sacrificed mouse with 70% ethanol.
3. Place mouse on a necropsy board and secure with dissecting pins. Use scissors and tweezers to open mouse and remove organs. Determining which organs should be collected is project dependent. However, it is recommended to always collect the spleen and liver as these organs often give important insight into why the mouse was moribund. Also collect the sternum for the potential analysis of bone marrow. In addition, it is always beneficial to collect any organ or tissue that appears abnormal.
4. Tissues and tumors that are collected should be divided into four sections. One section is fixed in 10% buffered formalin for 18 hr followed by 70% ethanol. This section can be used for H&E staining and/or IHC. The remaining three sections, which are generally 50 to 100 mg in size, are snap frozen in liquid nitrogen. These can be used for DNA, RNA and protein isolation.
5. Dispose of carcass and remaining tissues according to IACUC guidelines.

3. Isolating Genomic DNA from Tumors

Name of the reagent	Company	Catalogue number
Protein Precipitation solution	Qiagen	158910
Cell lysis buffer	Qiagen	158906
Proteinase K	Qiagen	158920
RNase A	Qiagen	158924
TE Buffer	Promega	V6232

Table 2. Reagents required.

1. Finely mince the frozen tumor sample (50 to 100 mg) using a clean razor blade.
2. Place minced tissue in a 1.5 ml microcentrifuge tube and add 1 ml of cell lysis buffer containing 5 µl Proteinase K solution (20 mg/ml).
3. Vortex thoroughly.
4. Incubate in a 55 °C shaker set at 250 rpm for at least 4 hr, preferably overnight.
5. Add 1 µl RNase A solution (10 mg/ml) and invert tube 25 times.
6. Incubate at 37 °C for 30 min.
7. Cool to room temperature by placing on ice for 3 min.
8. Add 333 µl Protein Precipitation solution and vortex vigorously.
9. Centrifuge at 14,000 rpm 10 min. Proteins should pellet. If pellet is very small, vortex again, incubate on ice for 5 min, and then re-centrifuge.
10. Pour off supernatant containing DNA into a clean 15 ml centrifuge tube.
11. Add equal volume of 100% isopropanol and mix by gently inverting 50 times.
12. Centrifuge at 3,500 rpm for 3 min.
13. Discard supernatant.
14. Add 1 ml of 70% ethanol and invert tube several times to wash the pellet.
15. Transfer the washed pellet along with the ethanol to a 1.5 ml microcentrifuge tube.
16. Centrifuge at 14,000 rpm for 5 min at 4 °C.
17. Pour off ethanol and invert tube to air dry (approximately 5 to 30 min).
18. Resuspend DNA pellet in 50 to 500 µl of TE depending on size of DNA pellet.

19. Optional incubation at 37 °C to resuspend DNA.
20. Store at 4 °C, or -20 °C for long term storage.

4. Linker Mediated-PCR Using Genomic DNA from Tumors

Name of the reagent	Company	Catalogue number
Bfal	New England Biolabs	R0568S
NlaIII	New England Biolabs	R0125S
MinElute 96 well plates	Qiagen	28051
QIAvac 96 Vacuum manifold	Qiagen	19504
Multi-MicroPlate Genie, 120V	Scientific Industries, Inc.	SI-4000
T4 DNA ligase with 5x ligation Buffer	Invitrogen	15224-041
BamHI	New England Biolabs	R0136S
25 mM dNTPs	Bioexpress	C-5014-200
Platinum Taq	Invitrogen	10966-034
FastStart Taq DNA Polymerase	Roche	12032929001
Agarose	Promega	V3121
TAE Buffer	Promega	V4271
TE Buffer	Promega	V6232
Ethidium bromide	Promega	H5041

Table 3. Reagents required.

Linker Sequences

Bfal linker + 5' GTAATACGACTCACTATAGGGCTCCGCTTAAGGGAC 3'

Bfal linker- 5' Phos-TAGTCCCTTAAGCGGAG 3'NH₂

NlaIII linker+ 5'GTAATACGACTCACTATAGGGCTCCGCTTAAGGGACCATG 3'

NlaIII linker- 5' Phos-GTCCCTTAAGCGGAGCC 3'

Primer Sequences

Primary PCR forward right side (NlaIII): Spl IRDR R1 1 GCTTGTTGAAGGCTACTCGAAATGTTTGACCC

Primary PCR forward left side (Bfal): Spl IRDR L1 1 CTGGAATTTTCCAAGCTGTTTAAAGGCACAGTCAAC

Primary PCR reverse for both right and left side: Spl Link1 1 GTAATACGACTCACTATAGGGC

Secondary PCR forward right side: Example of an Illumina barcoded secondary primer 5' AATGATACGGCGACCAACGAGATCTACACTCTTCCCTACACGACGCTCTTCCGATCTNNNNNNNNNNNNaGgtgtatgtaaactccgactcaa (The N's represent the 12 bp barcode, sequence in upper case are required for the Illumina platform, and the sequence in lower case will bind to the transposon.)

Secondary PCR forward left side: Example of an Illumina barcoded secondary primer 5'AATGATACGGCGACCAACGAGATCTACACTCTTCCCTACACGACGCTCTTCCGATCTNNNNNNNNNNNNaAgtgtatgtaaactccgactcaa (The N's represent the 12 bp barcode, sequence in upper case are required for the Illumina platform, and the sequence in lower case will bind to the transposon.)

Secondary PCR reverse for both right and left side: linker nested reverse primer 5' CAAGCAGAAGACGGCATACGAGCTCTTCCGATCTAGGGCTCCGCTTAAGGGAC

1. Anneal the + and - linkers for both Bfal linkers and NlaIII linkers by mixing 50 µl of linker+ (100 µM) with 50 µl of linker- (100 µM) and add 2 µl NaCl (5 M) in a 1.5 ml microcentrifuge tube. Place in 95 °C heat block for 5 min. Turn off heating block and let slowly cool to room temperature (this takes an hour or more). After cooling, annealed linkers may be stored in a -20 °C freezer until needed.
2. Prepare two aliquots of the tumor DNA, each aliquot containing 1 µg of DNA. One aliquot will be digested with a restriction enzyme that will cut close to the "right" end of the transposon. The second aliquot will be digested with a restriction enzyme that cuts close to the "left" end of the transposon. This is done to maximize the chances of amplifying every transposon insertion site.
3. Digest the tumor DNA in one aliquot using the "right" side enzyme NlaIII, and digest the tumor DNA in the other aliquot using the "left" side enzyme Bfal: combine 1 µl of enzyme, 5 µl 10x NEB Buffer #4, DNA (1 µg) and ddH₂O to a total volume of 50 µl. Digest DNA overnight at 37 °C in water bath or incubator.
4. Heat inactivate enzymes (Bfal = 80 °C for 20 min, NlaIII = 65 °C for 20 min).

5. Clean the digested samples by placing samples in MinElute 96 well plate, place plate in vacuum manifold and vacuum for about 15 min until wells look dry.
6. Remove plate from vacuum manifold and blot bottom of 96 well plate with paper towel to remove all liquid
7. Add 30 μ l ddH₂O to each well and shake on an orbital shaker set on high for 2 min, or pipette up and down 20 to 40 times.
8. Transfer entire volume in wells (30 μ l) from MinElute 96 well plate into clean 96 well plates.
9. Perform linker ligation reactions with digested DNA from step 4.8 and linkers from step 4.1. Mix 10 μ l digested DNA, 4 μ l 5x ligation buffer, 5.5 μ l annealed linkers (950 μ g/ μ l), 0.5 μ l T4 ligase (5 U/ μ l).
10. Incubate for 4 hr to overnight at 16 °C.
11. Heat inactivate the T4 DNA ligase at 65 °C for 10 min.
12. Clean up ligation using MinElute 96 well plates and a vacuum manifold as described in steps 4.5 - 4.6.
13. Resuspend in 30 μ l H₂O and transfer to a clean 96 well plate.
14. Perform a second DNA digestion in order to destroy remaining concatamer transposons. This is achieved by cutting the DNA a second time using an enzyme that cuts the plasmid vector DNA that was used to create the transgenic mice containing the transposon concatamer.
15. Digest DNA using BamHI (both left and right side): Mix 25 μ l of linker-ligated DNA from step 4.13, 5 μ l 10x NEB buffer #3, 0.5 μ l BamHI (20 U/ μ l), 0.5 μ l BSA (10 mg/ml), and 19 μ l ddH₂O.
16. Digest 3-6 hr or overnight at 37 °C.
17. Perform primary PCR using a primer specific for the transposon and a primer specific for the linker. These will vary depending upon what transposon and linker you are using. The primers listed in the primer table above work for the T2/Onc and T2/Onc2 transposons.
18. Primary PCR: 5 μ l 10x buffer, 2.0 μ l MgCl₂ (50 mM), 4 μ l dNTPs (2.5 mM), 1 μ l Primer 1 (20 μ M), 1 μ l Primer 2 (20 μ M), 0.25 μ l Platinum Taq (5 U/ μ l), 3 μ l Digested DNA with linkers (amount can vary), up to 50 μ l ddH₂O.
19. PCR Program: 94 °C for 5 min, 30 cycles of 94 °C for 30 sec, 60 °C for 30 sec, 72 °C for 90 sec. Final extension at 72 °C for 5 min.
20. Clean up PCR product using MinElute 96 well plates and a vacuum manifold as described above.
21. Resuspend in 30 μ l H₂O and transfer to a clean 96 well plate.
22. Make a 1:75 dilution of the 1° PCR by diluting 2 μ l of DNA from step 4.21 into 148 μ l ddH₂O
23. Perform secondary PCR using nested versions of the primers that also have the required sequence for the Illumina GAlx machine as well as a 12 base pair barcode that is unique for each tumor sample processed (see primer table above for examples of these primers).
24. Secondary PCR: 10 μ l 10x Buffer with MgCl₂, 8 μ l dNTPs (2.5 mM), 1 μ l Illumina barcoded secondary primer (20 μ M), 1 μ l Illumina Nest 1 secondary primer (20 μ M), 0.25 μ l FastStart Taq (5 U/ μ l), 2 μ l DNA from primary PCR diluted 1:75, up to 100 μ l ddH₂O.
25. PCR Program: 94 °C for 2 min, 30 cycles of 94 °C for 30 sec, 53 °C for 45 sec, 72 °C for 90 sec. Final extension at 72 °C for 5 min.
26. Run 45 μ l of this PCR reaction on a 1% agarose gel containing ethidium bromide (0.5 μ g/ml). There should be a "smear" of PCR products representing amplicons of the many different transposon insertions in the tumor (**Figure 3**).
27. Clean up remaining 50 μ l of PCR product using MinElute 96 well plates and a vacuum manifold as described above. Wash one time by placing 50 μ l ddH₂O in the wells and vacuuming again.
28. Resuspend in 30 μ l TE and transfer to a clean 96 well plate.
29. Determine the concentration of DNA in each sample. Combine equal amounts of DNA in a single 1.5 ml microfuge tube. This will be the library that is sequenced in a single lane of an Illumina HiSeq 2000 machine. It is possible to sequence several hundred tumors in a single lane of an Illumina run.
30. Submit the single tube containing equal amounts of DNA from all the tumors for sequencing. This can be done by your institution or commercially.

5. Analysis of Transposon Insertions

1. Software for analyzing transposon insertion data is available for free download via SourceForge (<http://sourceforge.net/projects/tapdancebio/>). The program, called TapDance, requires the sequence file from the Illumina platform and a barcode to library map text file.

Representative Results

After the breeding scheme has been established, breeders should produce a litter every 19-21 days. Litter size will vary between 5 and 12 pups, depending on the age of the breeders and the genetic background. In addition, make sure that the genotype of the litter follows Mendelian genetics as a way to confirm that the breeding scheme is both correct and successful.

For the experiment to be successful, tumor incidence in the experimental animals should be significantly greater than tumor incidence in the control animals. If no "predisposing" mutation was included in the experiment there should be few to no tumors in the control animals. If a "predisposing" mutation was included in the experiment, tumor incidence in these animals should be much lower than the incidence in animals with the mutation and SB activity.

LM-PCR should amplify hundreds of transposon insertions in a single tumor. After running half of the secondary PCR product on a 1% gel, there should be a "smear" of DNA representing this amplification (**Figure 3**). If there are only one or two bands, either SB was not active in the tumor or an error was made in the protocol (**Figure 4**).

Sequencing one to two hundred tumors in a single lane of the Illumina GAlx platform should produce over 30 million sequences of 100 bp each (**Figure 5**).

Table 1. Reagents required for section 2.

Table 2. Reagents required for section 3.

Table 3. Reagents required for section 4.

Figure 1. Flow chart showing the steps to carry out a forward genetic screen using the *Sleeping Beauty* system. First, a mouse model is generated. Mice are then necropsied when moribund and tumors are collected and isolated. Tumor DNA is purified and linker mediated-PCR performed. Finally, amplified tumor DNA with transposon insertions is sequenced and common insertion sites identified.

Figure 2. Breeding scheme to generate experimental cohort. Mice containing the transposase (Rosa26-LsL-SB11) are crossed to mice harboring the concatamer of transposons (T2-Onc). The offspring are then mated to mice carrying the designated tissue specific promoter driven *Cre Recombinase* (Cre).

Figure 3. Representative results of Secondary PCR. The expected product has a smear like appearance ranging from 100 to 600 base pairs.

Figure 4. If LM-PCR is unsuccessful, there will be an absence of the "smear" of DNA. Instead, you will see definitive bands of DNA.

Lane	Total Sequence #	Sequence Length	Total Sequence Bases
L1	46,466,558	101	4,693,122,358
L2	55,893,359	101	5,645,229,259
L3	55,815,774	101	5,637,393,174
L4	51,921,248	101	5,244,046,048
L5	50,406,233	101	5,091,029,533
L6	43,812,390	101	4,425,051,390
L7	56,522,031	101	5,708,725,131

Figure 5. Example data from a sequencing run that demonstrates a single lane of the Illumina GAIIx platform should produce over 30 million sequences of 100 base pairs each.

Discussion

A forward genetic screen using the *Sleeping Beauty* transposon system provides a method for identifying mutations that cause cancer. By selecting the appropriate promoter to control *Cre Recombinase* in addition to any predisposing mutations, the SB screen will identify known and novel candidate cancer genes.

The success of an SB screen is largely dependent on the mice selected for creating the screen. For the transposon, we recommend using two different mouse strains carrying the concatamer of oncogenic transposons on different chromosomes⁷. Careful thought should be used when choosing the tissue-specific *Cre Recombinase* that will activate the SB transposase. There should be evidence that the Cre enzyme is active in the cell type that is suspected of being the cell of origin of the cancer being modeled. Successful examples include Villin-Cre (GI tract cancer), Albumin-Cre (hepatocellular carcinoma), and Aid-Cre (lymphoma).^{8–10}

The number of mice required for a successful screen will depend upon the tumor penetrance. In general, more tumors and more mice will result in a more robust screen. To detect a 2-fold difference in cancer latency there should be at least 60 animals in both the control and experimental groups.¹¹

The versatile nature of these screens is powerful in respect to other techniques in that one can specify the nature of disease, follow the progression, and analyze the genetic composition. This technique has been successful in a number of screens that have yielded CIS lists that identify new potential cancer driving genes. With this information, directed studies may be performed to understand the function of these novel mutations. Overall, the use of this technique may lead to the eventual design of new drug therapies aimed to eliminate the results of these specific genetic mutations.

Disclosures

The authors have nothing to disclose.

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