Splinkerette Assay

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ABSTRACT

This protocol used to find the exact location of the integrated piggybac construct in the genome of stably transfected cell line. This assay determines the copy number of an exogenous gene in the established monoclonal piggybac cell line as wells as their insertion loci.

ATTACHMENTS

dh4dbiqa7.pdf

DOI

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PROTOCOL CITATION

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KEYWORDS

Splinkerette Assay, Piggybac construct, Monoclonal piggybac cell line

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MATERIALS TEXT

Materials

- Thermal cycler (PCR machine)
- Heat block
- Nanodrop

- Gel electrophoresis apparatus
- Non-CO2 incubator at 37 °C
- 4 °C -refrigerator
- Pipets
- PCR tubes and 1.5 Eppendorf tubes

Reagents

- **PiggyBac Splinkerette PCR Kit (Hera Biolabs, PB-SPLK-1)**

  Sau3AI - 200 units **New England**
- **Biolabs Catalog #R0169S** 5 U/µL

  EcoRV - 4,000 units **New England**
- **Biolabs Catalog #R0195S** 20 U/µL

  T4 DNA Ligase - 20,000 units **New England**
- **Biolabs Catalog #M0202S** 400 U/µL

  Taq 2X Master Mix - 500 rxns **New England**
- **Biolabs Catalog #M0270L**

  Epicentre QuickExtract™ DNA Extraction
- Solution **Epicentre Catalog #QE09050**
- **UltraPure DNase/RNase-free distilled water (Invitrogen, 10977-023)**
- QIAquick PCR purification kit
- QIAquick gel purification kit

  UltraPure™ Agarose **Invitrogen - Thermo**
- **Fisher Catalog #16500500**

  TOPO™ TA Cloning™ Kit for Sequencing, without competent cells **Thermo**
- **Fisher Catalog #450030**

  NEB 5-alpha Competent E. coli cells **New England**
- **Biolabs Catalog #C2987H**

Solutions preparation:

Prepare the Splinkerette Adaptor

A	B
50µM Adapter Primer Mixture	25 µL
10X Adapter Buffer	25 µL
Total 25µM Adaptor Mix	50 µL

Mix 25 µl from vial of 50 µl adapter mixture and 25 µl of 10X adapter buffer, that are included in the Splinkerette kit, in a PCR tube to the final concentration of [M]25 Micromolar (µM) . Heat the mixture to 95 °C for 00:05:00 , then cool by 1 °C every 00:00:15 until it reaches 24 °C . Afterwards, keep at 4 °C for future use.

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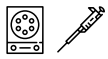
Harvest cells 4m

1 Passage and harvest the cells following the cell culture protocol.

100,000 cells would give enough genomic DNA.

2 Pellet the cells, aspirate the supernatant, and transfer the pellet to a **1.5 mL** Eppendorf tube.

3  4m

Add **1 mL** PBS and centrifuge at **400 x g** for **00:04:00** .

4 Remove the supernatant very carefully.

The pellet is very loose now.

5 Snap freeze the pellet and store it at **-80 °C** for later genomic DNA extraction.

Genomic DNA extraction 8m

6 

Add **50 µl** quick extract (QE) reagent to the pellet and vortex well. Aliquot QE reagent in **1.5 mL** tubes and store at **-20 °C** .

7 Heat the tube of cell pellet in QE at **65 °C** for **00:06:00** . 6m

8 Next, heat at **98 °C** for **00:02:00** . 2m

9 

Vortex well to dissolve the pellet completely.

10 Measure the DNA concentration using a nanodrop.

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Sau3AI digestion 22m

11

Set all reactions from here to the end with a positive control alongside the gDNA prepared above.

You may use the piggybac plasmid construct that was transfected into the cells for positive control.

A	B
Genomic DNA	2 µg
Sau3AI enzyme (5-20 units)	1 µL
10X cutsmart buffer	3 µL
DNase/RNase-free H2O to final volume	30 µL

Set the digestion reaction as listed in a **1.5 mL** tube.

12 2m

Incubate at **37 °C** in an incubator or heat block **Overnight** .

13 20m

Heat inactivate the reaction at **65 °C** for **00:20:00** .

14

Purify Sau3AI digested gDNA using PCR purification kit following manufacturer protocol. Elute DNA in **30 µl** H_2O.

15 Store the tube at **-20 °C** for later.

Ligation of adaptors 20m

16

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A	B
Sau3A1-digested gDNA (from step 6.3)	300 ng
Adaptor mix (25 µM) (prepared above)	1 µL
10X T4 DNA ligase buffer	4 µL
T4 ligase	1 µL
DNase/RNase-free H2O to final volume	40 µL

16.1

Incubate the ligation reaction at **4 °C** **Overnight** (12 to 16 hours).

20m

16.2 Heat inactivate the reaction at **65 °C** for **00:20:00** .

16.3 Cool down the sample to **Room temperature** .

EcoRV digestion 20m

17

A	B
Ligation reaction (from step 6.4)	40 µL
EcoRV enzyme	1 µL
10X cutsmart buffer	10 µL
DNase/RNase-free H2O	49 µL
Final volume	100 µL

Set the digestion reaction as listed in a **1.5 mL** tube.

20m

18

Incubate at **37 °C** in an incubator or heat block **Overnight** .

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19 Purify EcoRV digestion reaction using PCR purification kit following manufacturer protocol. Elute DNA in **32 µl** TE buffer.

20 Store the tube at **-20 °C** for later.

Spkt1 PCR

21 Set up the primary PCR amplification in a PCR tube.

A	B
Taq 2X Master Mix	10 µL
Spkt-Primer Mix-1	1.5 µL
Template (from step 6.5.3 elute)	5 µL
DNase/RNase-free H2O	3.5 µL
Final volume	20 µL

22 Run the reaction in the thermal cycler with the following program for Taq 2X master mix polymerase.

A	B	C	D
Initial denaturation	95°C	3 min	1X
Denaturation	95°C	30 sec	34X
Annealing	55°C	30 sec	
Extension (1min/kb)	68°C	2 min	
Final extension	68°C	5 min	1X
Hold	4°C	Forever	

23 Store PCR product at **-20 °C** .

Spkt2 nested PCR

24 Set up the secondary nested PCR in a PCR tube.

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A	B
Taq 2X Master Mix	10 µL
Spkt-Primer Mix-2	1.5 µL
Template (from step 6.6.3)	1 µL
DNase/RNase-free H2O	7.5 µL
Final volume	20 µL

25 Run the reaction in the thermal cycler with the following program for Taq 2X master mix polymerase.

Annealing temperature and final extension time are different from the 1st PCR.

A	B	C	D
Initial denaturation	95°C	3 min	1X
Denaturation	95°C	30 sec	34X
Annealing	57°C	30 sec	
Extension (1min/kb)	68°C	2 min	
Final extension	68°C	20 min	1X
Hold	4°C	Forever	

26 Store PCR product at **-20 °C** .

Topo TA cloning and transformation 4h 7m 45s

27 Run the PCR product from step 28 on 1.5% agarose gel.

The number of bands would indicate the number of integration sites if more than one copy is integrated. It may also indicate the polyclonal nature of the cell line with different integration locus in each clone. The positive control plasmid should show only one band.

28 Gel purify each band separately. Store at **-20 °C** .

However, fresh product would be more efficient for Topo TA cloning.

29 Set up ligation reaction in PCR tube as following:

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A	B
Gel-purified PCR product (from step 6.8.2)	0.8 µL (0.4-1.6 µL)
Salt solution	0.4 µL
Topo vector	0.4 µL
DNase/RNase-free H2O	0.8 µL
Final volume	2.4 µL

30  30m

Incubate the reaction at **Room temperature** for less than **00:30:00** .

31 Transfer to ice or store at **-20 °C** for later use.

32  30m

Mix gently **1 µl** ligation reaction with **20 µl** competent cells in **1.5 mL** tube **On ice** . Incubate **On ice** for **00:30:00** .

33 Heat shock at **42 °C** for **00:00:45** . Leave **On ice** for **00:02:00** - **00:05:00** . 7m 45s

34  1h

Add **200 µl** **Room temperature** SOC medium to the transformed cells. Incubate at **37 °C** in an orbit shaker for **01:00:00** .

35  1h

Plate **20 µl** and **100 µl** on pre-warmed agar plate supplemented with **100 µg/ml** ampicillin. Incubate at **37 °C** **Overnight** .

36  1h

Pick a few colonies from each plate. Inoculate into **5 mL** LB broth supplemented with **100 µg/ml** ampicillin. Incubate at **37 °C** in an orbit shaker **Overnight** .

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37 Extract the DNA using Qiagen DNA miniprep.

Sequencing

38

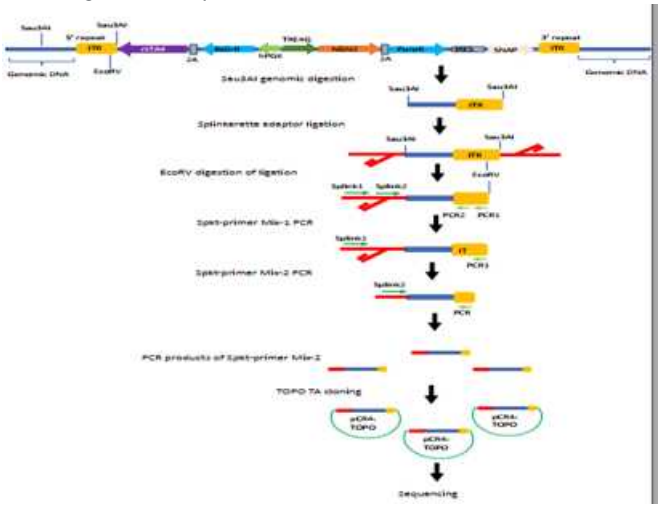
Topo TA cloning kit uses vector pCR4 for cloning. Primers M13 reverse and M21 forward have binding sites on either side of the cloning site on the vector. We only need to sequence with one of these primers. Though the primers are provided in the TOPO TA cloning kit you may submit the samples without them because sequencing facilities have these primers, too. Follow the guidelines on submitting samples for sequencing provided on vendor's website.

Provide **10 µl** volume of plasmid prep from step 39 in PCR tube. The final concentration needs to be **80 ng/µl** - **100 ng/µl** .

39 Fill the submission form on vendor's website. Choose their relevant in-house primer from their primer list and mention on the form.

The sequencing result would show the genomic sequence right before the 5' ITR end of the piggybac construct as well as part of the construct.

40 Blast the genomic sequence on NCBI website for the exact locus the piggybac construct has integrated.



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