

Inverse PCR

Gep-SD5/Gen-SD5 (5' Flanking)

A. Genomic DNA Isolation from 96-well Plates and Restriction Analysis

1. Aspirate media and wash twice with PBS
2. Add 40 μ l tail buffer with 0.5 mg/ml proteinase K
3. Seal with parafilm and incubate at 56°C overnight in lunch box container
4. Add 80 μ l *freshly* prepared NaCl/EtOH mix to each well (15 μ l 5M NaCl per ml EtOH)
5. Let sit undisturbed for 1-2 hours at room temperature
6. Lay paper towels over the wells and *slowly* invert
7. Wash 3X with 70% EtOH at room temp, slowly inverting as before
8. Air dry
9. For first replicate add 50 μ l TE, wrap with parafilm and store at 4°C
10. For second replicate add 35 μ l restriction enzyme mix to each well

385 μ l	192.5 μ l	10X buffer
4 μ l	2 μ l	100mM spermidine
55 μ l	27.5 μ l	RNAse A
26 μ l	13 μ l	100U/ μ l <i>Hind</i> III
<u>3380μl</u>	<u>1690μl</u>	DDW
3850 μ l	1925 μ l	

11. Seal with parafilm and incubate overnight at 37°C in humidified lunch box container
12. Run 15 μ l on a 0.8% agarose gel at 18-20V overnight to check digests

B. Ligation

1. Add 100 μ l DDW to 2 μ l *Hind*III digested DNA and heat-inactivate at 65°C for 30 minutes
2. Cool to 4°C
3. Add 300 μ l of ligation mix:

40 μ l	10X ligation buffer (total = 1ml):
	400 μ l 1M Tris-Cl, pH7.8
	100 μ l 1M MgCl ₂
	100 μ l 1M DTT
	400 μ l DDW
20 μ l	100mM ATP
188 μ l	DDW
2 μ l	T4 DNA ligase (2U)

4. Incubate overnight at 4°C
5. Add 80µl 3M NaOAc, pH5.2, 1µl 20mg/ml glycogen and 900µl EtOH and place in -20°C ≥ 3 hrs
6. Centrifuge 15 minutes at 14K
7. Wash, dry, and resuspend pellet in 100µl TE

C. PCR

1. Distribute 10µl aliquots of ligation products to wells of a 96-well PCR plate
2. Add 15µl mix:

110µl	10mM dNTPs
55µl	10µM AdiPCR1F
55µl	10µM AdiPCR1R
275µl	10 x Taq buffer
35µl	Taq polymerase
<u>1120µl</u>	DDW
1650µl	

3. Add mineral oil to the outer wells as well as the four corner B2, B11, G2 and G11
4. Seal plate with adhesive foil
5. Use INVPCR1 program:

- i. 94°C, 2 min
- ii. 40 cycles (94°C, 30 sec; 61°C, 30 sec; 72°C, 4 min)
- iii. 72°C, 4 min

6. Add 100µl of DDW to dilute primary PCR products and then transfer 1µl of this to a second 96 well plate for nested PCR.
7. Add 50µl mix:

220µl	10mM dNTPs
110µl	10µM AdiPCR1Fnest
110µl	10µM Ad/HPRTiPCRnestR
550µl	10 x Taq buffer
60µl	Taq polymerase
<u>4450µl</u>	DDW
5500µl	

8. Use INVPCR2 program:

- i. 94°C, 2 min
- ii. 40 cycles (94°C, 30 sec; 61°C, 30 sec; 72°C, 3 min)
- iii. 72°C, 4 min

9. Analyze 10µl on a 1.8 % agarose gel

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10. PCR products >300 bp can be sent for direct sequencing using the LTRseq1 primer.

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pSD5/pGT6/pGT7/pGT8 (5' Flanking)

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18. Lay paper towels over the wells and *slowly* invert
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18. Use INVPCR2 program:

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- ii. 40 cycles (94°C, 30 sec; 61°C, 30 sec; 72°C, 3 min)
- iii. 72°C, 4 min

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