Splinkerette Protocol

Adapted from the protocol in Horn et al., (Nature Genetic 2007).
Thanks to Dr. Noppinger and Dr. Irgang for supplying us with the original protocol.

1. Genomic DNA (gDNA) preparation from ES cells

DNA isolation lysis buffer: (For 50mls)
10mM Tris/HCl pH 7.5 (500ul of 1M Tris)
10mM EDTA (1ml of 0.5M EDTA)
10mM NaCl (100ul of 5M NaCl)
0.5% SDS (2.5ml of 10% SDS)
1mg/ml Proteinase K (to be added fresh, 50ul of 20mg/ml stock per ml of lysis buffer)

1.1. Trypsonize cells and spin down. Wash pellet once with PBS and collect pellet in a 1.5ml tube.
1.2. Add 450µl of lysis buffer to each tube
1.3. Incubate at 55-60°C for 2-3 hours
1.4. Cool down to room temperature, spin-down condensed liquid (20°C, 2min, 2000g)
1.5. Add 200µl of potassium acetate. Mix well and let sit for 1-2 minutes.
1.6. Spin in centrifuge at 12,000rpm for 3-5 minutes. You should see a large pellet of protein at the bottom of your tube.
1.7. Remove supernatant and transfer to fresh tubes (should get approx. 500ul).
1.8. Add at least 700-800ul of isopropanol to the supernatant (amount of isopropanol should be about 1.5-2x the amount of supernatant).
1.9. Invert tube several times, you should see strands of DNA precipitate out of the solution.
1.10. Spin in centrifuge at 12,000rpm for 2 minutes. You should see a small pellet at this point.
1.11. Remove and discard supernatant, being very careful not to discard the pellet.
1.12. Add 800ul 70% ethanol. Gently invert tube several times to wash the pellet.
1.13. Spin in centrifuge at 12,000rpm for 2 minutes.
1.14. Remove and discard the supernatant, pellet will be loose so be careful not to lose the pellet.
1.15. Repeat steps 1.11 – 1.14 again.
1.16. Let the pellet air dry (at least 15-20min.) by leaving the cap of the tube open.
1.17. Resuspend in 50ul to 100ul of TE (depending on amount of cells used).
1.18. Incubate at 55°C for 2 hours to help dissolve and then store at 4^{o}.

2.0 Restriction digest and ligation

2.1 Prepare restriction master mix (final reaction volume: 20 µl)

For GepNMDi and UPA vectors

Component	for 1 sample
10x NEB buffer 3	2.0µl
100x BSA (NEB)	0.2µl
*Apo*I (NEB, 10U/µl)	0.5µl
Bidest-water	8.3µl

2.2. Pipette 11.0µl of restriction mix to each well of a PCR plate

2.3. Transfer 9.0µl of dissolved gDNA to each well

2.4. Incubate:
2.5 h at 50°C
20 min at 80°C
Cool down to 4°C

Preparation of Splinkerette adaptor

2.5. While the restriction digest is occurring, prepare the splinkerette adaptor.

Component	for 1 sample
Primer SpAa, 10µM:	0.15µl
Primer SpBb, 10µM:	0.15µl
SuRE buffer M (Roche)	0.05µl
PCR water:	0.65µl

2.6. Incubate Splinkerette mixture for 5 min at 97°C. Turn off heating block and let tube cool in block to room temperature to allow annealing.

Ligation of Adaptor to Digested DNA

2.7. Prepare ligation master mix as follows (final reaction volume will be 30µl)

Component	for 1 sample
Splinkerette:	1.0µl
10x DNA Ligation buffer	3.0µl
T4-DNA Ligase (400U/µl)	1.0µl
Bidest-water	5.0µl

2.8. Pipette 10.0µl ligation mix directly to each tube of digested DNA, mix well.
2.9. Incubate over night (12-16h) at 16°C.

3.0 Purification of Ligated Products

3.1 Use a PCR purification kit to purify the ligated products.

4.0 First round PCR

For GepNMDi3 and GepSD5 clones

Primer pair for 5' side: Primer 1 = Sp0F Primer 2 = AdiPCR1R

Primer pair for 3' side: Primer 1 = Sp0F Primer 2 = Spl3Gep4F1

For UPA clones

Primer pair for 5' side: Primer 1 = Sp0F Primer 2 = Spl5-UPA-R1

Primer pair for 3' side: Primer 1 = Sp0F Primer 2 = Spl3-UPA-1F3

Master Mix

Component	for 1 sample
PCR water	15.05ul
10x rxn buffer (- MgCl)	2.5ul
50mM MgCl2	0.875ul
10mM dNTP mix	0.5ul
primer 1 (10uM)	0.5ul
primer 2 (10uM)	0.5ul
Taq Polymerase (5 U/µl)	0.1ul
gDNA	5.0ul

Final volume is 25ul

1st PCR program

94°C for 2min
3 cycles of (94°C/20 sec, 64°C/15sec, 72°C/5 min)
30 cycles of (94°C/20sec, 58°C/15sec, 72°C/3 min)
72°C for 10min
4°C hold

5.0 Second round PCR

For GepNMDi3 clones

Primer pair for 5' side: Primer 1 = Sp1F Primer 2 = iPCR4nestF

Primer pair for 3' side: Primer 1 = Sp1F Primer 2 = Spl3Gep2FII

For UPA clones

Primer pair for 5' side: Primer 1 = Sp1F Primer 2 = iPCR4nestF

Primer pair for 3' side: Primer 1 = Sp1F Primer 2 = Spl3Gep2FII

5.1 **Dilute the 1st PCR product by adding 50-100ul of PCR water to each tube and mixing.

Master Mix

Component	for 1 sample
PCR water	19.05ul
10x rxn buffer (- MgCl)	2.5ul
50mM MgCl2	0.875ul
10mM dNTP mix	0.5ul
primer 1 (10uM)	0.5ul
primer 2 (10uM)	0.5ul
Taq Polymerase (5 U/µl)	0.1ul
Diluted 1st PCR	1.0ul

Final volume is 25ul

2nd PCR program

94°C for 2min
35 cycles of (94°C/20 sec, 60°C/15sec, 72°C/3 min)
72°C for 5min
4°C hold

5.2 Analysis the second round PCR product on a 1.5% gel before sending for sequencing.

Sequencing Primers

GepNMDi3 and GepSD5	5' iPCR4nestF	3' Spl3Gep2FII
UPA	5' iPCR4nestF	3' Spl3-UPA-F2

Primers for Splinkerette

Common for all clones

SpAa_Apo1: (for splinkerette adaptor)
CGAAGAGTAACCGTTGCTAGGAGAGACCGTGGCTGAATGAGACTGGTGTCGACACTAGTGG

SpBb_ApoI: (for splinkerette adaptor)
AATTCCACTAGTGTCGACACCAGTCTCTAATTTTTTTTTTCAAAAAAA

Sp0F: (for first round PCR)
CGAAGAGTAACCGTTGCTAGGAGAGACC

Sp1F: (for second round PCR)
GTGGCTGAATGAGACTGGTGTCGAC

Vector specific primers for PCR and Sequencing

AdiPCR1R	TCATCAAGGAAACCCTGGAC
iPCR4nestF	TTGTGGTCTCGCTGTTCCTT
Spl3Gep4F_I	GCAGGCGCATAAAATCAGTC
Spl3Gep2F_II	TGACGGGTAGTCAATCACTCAG
Spl5-UPA-R1	CGACGGTATCGATTAGTCCAA
Spl3-UPA-1F3	GGTGTTCCGAACTCGTCAGT
Spl3-UPA-F2	TTCCATCTGTTCCTGACCTTG

References:

This protocol has been modified from the original one found in:

Horn C, Hansen J, Schnütgen F, Seisenberger C, Floss T, Irgang M, De-Zolt S, Wurst W, von Melchner H, Noppinger PR. Splinkerette PCR for more efficient characterization of gene trap events. Nat Genet. 2007 Aug;39(8):933-4. Erratum in: Nat Genet. 2007 Dec;39(12):1528. PMID: 17660805