SPLINKERETTE Protocol

Steps 1-3 are currently conducted in 96-well microtiter plates whereas steps 4-6 are carried out in 384-well format.

1. Genomic DNA (gDNA) preparation from frozen ES cell clone pellets

Lysis buffer:
10mM Tris/HCl pH 7.5
10mM EDTA
10mM NaCl
0.5% SDS
1mg/ml Proteinase K (to be added freshly)

1.1. starting material: A 96-well microtiter plate containing PBS-washed frozen ES cell clone pellets
1.2. add 40 µl lysis buffer to each well
1.3. seal 96-well plate tightly (e.g. using Microseal A Film [Biozym, Art.Nr. 621715])
1.4. incubate at 60°C for 2h
1.5. cool down 96-well plate to room temperature, spin-down condensed liquid (20°C, 2min, 4000g)
1.6. add 80 µl icecold 75mM (w/v) NaCl/EtOH suspension
1.7. incubate 1 h at room temperature for gDNA precipitation
1.8. slowly invert plate over a sink to discard the ethanolic solution. The precipitated nucleic acid should remain attached to the base of the wells and may be visible against bright light.
1.9. allow the remaining ethanol to drain from the plate on a bed of paper towels
1.10. wash gDNA pellet 3x with 150 µl 70% EtOH each, being careful not to dislodge the precipitate
1.11. dry gDNA pellet in speed-vac for 5-10min until the last traces of ethanol have evaporated
1.12. elute genomic DNA with 40 µl 5mM Tris/HCl pH 8.0
1.13. dissolve gDNA over night (12-16 h) at room temperature

2. Restriction digest and ligation

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

2.1.1. for FlipROSAbetageo vectors (V08. V09, V10, V11, V12, V13, V14):

	for 1 sample
10x NEB buffer 2	2.0 µl
*Bst*YI (NEB, 10U/µl)	0.5 µl
Bidest-water	8.5 µl

2.1.2. for ROSAbetageo vectors (V04, V16, V17):

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

2.2. pipet 11.0 µl restriction mix to each sample

2.3. transfer 9.0 µl of gDNA to each sample

2.4. incubate:
2.5 h at 60°C
20 min at 80°C
cool down to 4°C

2.5. prepare Splinkerette:

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

2.6. incubate Splinkerette mixture for 4 min at 97°C
slowly let cool down on benchtop for annealing

2.7. prepare ligation master mix as follows (final reaction volume: 30 µl)

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

2.8. pipette 10.0 µl ligation mix to each sample

2.9. incubate over night (12-16h) at 16°C

3. Ligated genomic DNA purification using Millipore-Multiscreen-PCR-Filter-plates

3.1. add 25 µl Bidest-water to Multiscreen-PCR-plate to wet the membrane

3.2. dilute the 30 µl gDNA with 150 µl Bidest-water and transfer it into the Multiscreen-PCR-plate

3.3. place the Multiscreen PCR-plate on top of the Multiscreen-manifold, place a plate for waste

3.4. apply vakuum at 15 inches for 10 min

3.5. add 40 µl 5mM Tris pH 8.0

3.6. retrieve clean ligated DNA by pipetting up and down several times

3.7. transfer cleaned DNA into a new 96-well plate

3.8. merge cleaned DNA of 4 x 96-well plates to 1 x 384-well plate
by transfering 5 µl of clean DNA according to the following pipetting scheme:

384 well A01 corresponds to 1st 96 well A01

384 well A02 corresponds to 2nd 96 well A01

384 well B01 corresponds to 3rd 96 well A01

384 well B02 corresponds to 4th 96 well A01

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3.9. continue to first round PCR

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4. First round PCR

primer pair for 5' side: Sp0F/5'Sp0R

primer pair for 3' side: Sp0F/3'Sp0R

component	amount (µl)
gDNA	5.00
sterilized, destilled water	6.17
10x reaction buffer (incl. 1.5mM Mg^{2+})	1.50
25mM $MgCl_2$	0.15
2mM dNTP mix	1.50
primer Sp0F (10pmol/µl)	0.30
primer Sp0R (10pmol/µl)	0.30
Eppendorf Taq Polymerase (0.04 U/µl)	0.08
final volume	15.00

program: 94°C/75s + 2x(94°C/20s; 64°C/15s) + 30x(94°C/20s; 58-64°C/15s; 72°C/120s) + 72°C/7mins

5. Second round PCR

primer pair for 5' side: Sp1F/5'Sp1R

primer pair for 3' side: Sp1F/3'Sp1R

component	amount (µl)
first round PCR product	1.00 (or minute amount by stamp)
sterilized, destilled water	6.42
10x reaction buffer	1.00
25mM MgCl2	0.10
2 mM dNTP mix	1.00
primer Sp1F (10pmol/µl)	0.20
primer Sp1R (10pmol/µl)	0.20
Taq Polymerase (Eppendorf)	0.08
final volume	10.00

program: 94°C/60s + 30x(94°C/20s; 60°C/15s; 72°C/60s) + 72°C/7mins

analyze 2-3µl of secondary amplification product on 1.4% agarose gel or proceed directly to sequencing

6. BigDye Terminator Sequencing PCR

component	amount (µl)
second round PCR product	1.00
sterilized, destilled water	7.00
seq. primer (10pmol/µl)	1.00
BigDye Terminator Mix	1.00
final volume	10.00

program: 96°C/60s + 25x(96°C/10s; 55°C/5s; 60°C/240s)

7. Primers

Number	Sequence (5´ -> 3´)	Primer type	Tm value
7.1. ROSAbetageo vectors (V04, V16, V17)			
40	CGAAGAGTAACCGTTGCTAGGAGAGACCGTGGCTG AATGAGACTGGTGTCGACACTAGTGG	Splinkerette SpAa	
62	AATTCCACTAGTGTCGACACCAGTCTCTAATTTTTTT TTTCAAAAAAA	Splinkerette SpBb	
38	CGAAGAGTAACCGTTGCTAGGAGAGACC	Sp0F	62°C
56	AGGAAACCCTGGACTACTGC	5' Sp0R	62°C
58	GACACAGATAAGTTGCTGGC	3' Sp0R	60°C
39	GTGGCTGAATGAGACTGGTGTCGAC	Sp1F	61°C
47	GATGGAACAGCTAGAGAACC	5' Sp1R	60°C
43	CTCAGTTATGTATTTTTCCATGCCTTGC	3' Sp1R	59°C
21	TTGTGGTCTCGCTGTTCCTTGGG	5' Seq	62°C
37	GCTAGCTTGCCAAACCTACAGGTGG	3' Seq	61°C
7.2. FlipROSAbetageo vectors (V08. V09, V10, V11, V12, V13, V14)			
40	CGAAGAGTAACCGTTGCTAGGAGAGACCGTGGCTG AATGAGACTGGTGTCGACACTAGTGG	Splinkerette SpAa	
41	GATCCCACTAGTGTCGACACCAGTCTCTAATTTTTT TTTTCAAAAAAA	Splinkerette SpBb	
38	CGAAGAGTAACCGTTGCTAGGAGAGACC	Sp0F	62°C
33	CGACCAGCTGTGCGCATAGTG	5' Sp0R	59°C
35	AGTCATAGACACTAGACAATCGG	3' Sp0R	60°C
39	GTGGCTGAATGAGACTGGTGTCGAC	Sp1F	61°C
34	TTTGGCAAGCTAGCACAACC	5' Sp1R	60°C
36	CAGTCAATCGGAGGACTGGCG	3' Sp1R	59°C
21	TTGTGGTCTCGCTGTTCCTTGGG	5' Seq	62°C
37	GCTAGCTTGCCAAACCTACAGGTGG	3' Seq	61°C