Haplobank

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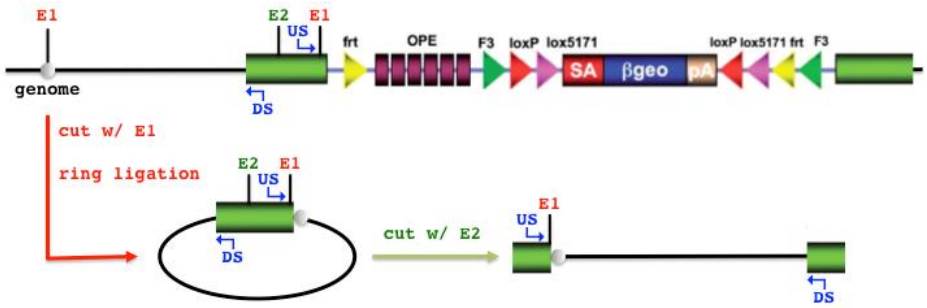
INVERSE PCR - PCR STRATEGY TO MAP GENOMIC INTEGRATION SITES OF GENE TRAP VECTORS

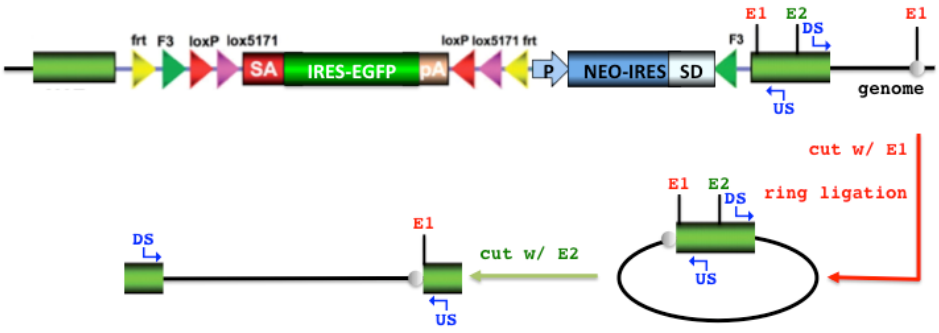
Enzyme1 (E1) is used to fragment the genome. As the recognition sequence for E1 is also present in the terminal repeat of the gene trap vector, it is possible to retrieve the exact integration site of the gene trap cassette within the genome by circularizing E1-digested gDNA (ring ligation) and subsequently amplifying the genomic region by inverse PCR (iPCR) using universal primers "US" and "DS". To improve iPCR efficiency, a linearization step using E2 was introduced, which re-opens the rings. Moreover, each integration site can be mapped by using two different Enzyme1s. We would recommend splitting the sample in two and using both enzymes E1 individually.

Please keep in mind the "directionality" of the mapping strategies for different mutagens, as this affects the assignment of a particular mutation to the sense and anti-sense strand in the genome, respectively.

Mapping - 5' strategy (Lenti-EGT, Retro-EGT)



Mapping - 3' strategy (Tol2-EGT, Tol2-polyA)



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MUTAGENISIS SYSTEMS	Enzyme 1 (E1)		Enzyme 2 (E2)	Mapping Strategy
Lenti-EGT	NlaIII	TaqI	PacI	5´
Retro-EGT	NlaIII	MseI	SbfI	5´
Tol2-EGT	NlaIII	TaqI	PacI	3´
Tol2-polyA	NlaIII	TaqI	PacI	3´

iPCR PRIMER SEQUENCES

DS: GAGCCAGAACCAGAAGGAACTTGAC
US: GTGACTGGAGTTCAGACGTGTGCTCTTC

REAGENTS AND BUFFERS

RNAse A	Qiagen, 19101 (100mg/ml)
Roti-Phenol/Chloroform/Isoamyl alcohol	Roth, A156.2
mixture of Chloroform:Isoamyl alcohol 24:1	Sigma, Merck
Optional: Phase Lock Gel	VWR 733-2478
Isopropanol, 70% Ethanol, TE Buffer	
Picogreen	Invitrogen, P7589
Restriction enzymes and buffers	New England Biolabs
T4 DNA ligase and buffer	Roche Applied Science
PCR kit (polymerase and buffer)	
QIAquick PCR Purification Kit or	Qiagen, 28106
Sera-Mag SpeedBeads	GE 11829912
illustra ExoProStar 1-Step Kit	GE 11911411

Preparation of Genomic DNA lysis buffer (GDLB):

10mM Tris-HCl pH 8.0
5mM EDTA
100mM NaCl
1.0% SDS
1 mg/ml Proteinase K
→ autoclave

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iPCR PROTOCOL

1. **Genomic DNA prep**
   - Lyse cell pellet according to pellet size in 0,5-5mL GDLB
   - Incubate at 55˚C, over night
   - Add 1:1000 RNAse A (100mg/ml) for ~1h at 37˚C
   - 2x Phenol/Chloroform/Isoamyl alcohol extractions (using Phase Lock Gel)
   - 1x Chloroform/Isoamy alcohol extraction (using a phase lock system)
   - Isopropanol precipitation of gDNA
   - Wash gDNA pellet with 70% EtOH
   - Resuspend in 0,05-1mL TE Buffer
   - Quantify DNA using Picogreen

2. **Digestion of samples with enzymes E1 in parallel (per sample two seperate reactions!)**

gDNA 100ng/µl	10 µl
10x CutSmart	8 µl
Enzyme1	3 µl
dH2O	59 µl
	80 µl

→ incubate 37˚C or 65˚C (TaqI), over night

2. **Purify restriction digests (Qiagen PCR Purification Kit or Sera-Mag SpeedBeads), elute in 50µl in total**

3. **Ring ligation**

E1-gDNA	50 µl
10x LB	30 µl
T4 DNA Ligase	2 µl
dH2O	218 µl
	300 µl

→ incubate 16˚C, over night

3. **Heat-inactivate T4 DNA Ligase at 65˚C for 15min**

4. **Linearize samples with enzyme E2 at 37˚C for 2h**

RL-E1-gDNA	300 µl
Enzyme2	1 µl

4. **Purify restriction digest (Qiagen PCR kit or Sera-Mag SpeedBeads), elute in 50µl in total**

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5. iPCR reaction

E2-RL-E1-gDNA from 7.	10 µl
primer US 100 µM	0.1µl
primer DS 100 µM	0.1µl
10 mM dNTP mix	1 µl
10x Polymerase Buffer	5 µ
20x Polymerase	3 µl
dH2O	30.8µl
	50 µl

Biorad C1000 cycle parameters:

95deg, 3’	
95deg, 15”	37x
61deg, 25”	
72deg, 75”	
72deg, 5’	

- Analyze 20µl on an agarose gel
- Purify PCR products using the QIAquick kit or the ExoStar kit for Sanger Sequencing