



Standard Operating Procedure

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Protocol:	ESC04	Version:	1.0
Protocol Section:	Embryonic Stem Cells	Effective Date:	25-Apr-2008
Protocol Title:	Splinkerette PCR amplification of gene trap sequence tags from single clones	Date Reviewed:	23-Apr-2008
		Date Revised:	13-Jun-2008

This standard operating procedure (SOP) was adapted from the splinkerette protocol in Horn *et al.* (Nature Genetics, 2007) by Tammy Reid at the Centre for Modeling Human Disease (CMHD) Gene Trap Core. This SOP describes the protocol for splinkerette amplification of genomic DNA adjacent to gene trap vectors Gep-NMDi3 and UpATrap used at the CMHD.

Due to the high throughput nature of gene trap ES cell clone production it is advisable to confirm the trapped gene by sequence tag confirmation following clone expansion for distribution. This protocol describes the process for gene trap sequence tag confirmation by splinkerette PCR. In the case of clones originally mapped with 3'-RACE tags, splinkerette PCR provide genomic localization of the inserted clone.

Related Protocols:

Protocol ESC01 describes the materials and SOPs for the cryorecovery and expansion of ESC clones.

Materials:

As for Protocol ESC01

DNA isolation buffer [10 mM Tris-HCl, pH 7.5; 10 mM EDTA; 10 mM NaCl; 0.5% (w/v) SDS plus 1 mg/ml proteinase K added immediately before use]

1.5 ml microcentrifuge tubes, DNase-free

55-60°C heat block, water bath, or incubator

5M potassium acetate [60 ml KOAc; 11.5 ml glacial acetic acid; 28.5 ml water to give 3M with respect to potassium and 5 M with respect to acetate; from Sambrook]

2-propanol, DNase-free

70% (v/v) ethanol, DNase-free

TE buffer [10 mM Tris-HCl, pH 7.5; 1 mM EDTA]

ApoI restriction enzyme and digestion buffers [NEB, Cat. No. R0566]

PCR water [e.g. Invitrogen, Cat. No. 10977-015]

PCR machine

Primers for splinkerette ligation and PCR [see Section 7. Primer Sequences]

DNA ligase and ligation buffer [e.g. Invitrogen, Cat. No. 15224-017]

Taq polymerase and PCR buffers [e.g. Invitrogen, Cat. No. 10966-018]

Equipment and reagents for DNA agarose gel electrophoresis

Protocol:*Section I. Genomic DNA (gDNA) isolation from ES cells:*

- 1.1 Passage cells on gelatinized plates, without feeders, at least once.
This reduces the contamination of the isolated gDNA with wild-type DNA from feeders.
- 1.2 Trypsinize cells and collect by centrifugation. Aspirate media and wash pellet once with 1X PBS. Resuspend pellet in small volume of 1X PBS, transfer to a 1.5 ml DNase-free microfuge tube and collect cells by centrifugation. Aspirate supernatant.
- 1.3 Add 450µl of DNA isolation buffer to each tube and resuspend cell pellet by gentle pipetting.
- 1.4 Incubate at 55-60°C for 2-3 hours.
- 1.5 Allow to cool to room temperature, centrifuge to collect condensed liquid (20°C, 2min, 2000 xg).
- 1.6 Add 200 µl of potassium acetate. Mix well and let sit at room temperature for 1-2 minutes.
- 1.7 Collect precipitate by centrifugation at 12,000 rpm for 3-5 minutes.
A large pellet of precipitated SDS and protein/debris should be seen.
- 1.8 Remove supernatant and transfer to fresh tube (~500 µl).
- 1.9 Add at least 700-800 µl of isopropanol to the supernatant.
Volume of isopropanol should be about 1.5-2X the volume of supernatant.
- 1.10 Invert tube several times to precipitate the DNA.
Strands of DNA precipitate should become visible.
- 1.11 Centrifuge at 12,000 rpm for 2 minutes to pellet the DNA.
Pellet will be small.
- 1.12 Remove and discard supernatant, being very careful not to dislodge the pellet.
- 1.13 Add 800 µl 70% ethanol. Gently invert tube several times to wash the pellet.
- 1.14 Centrifuge at 12,000 rpm for 2 minutes.
- 1.15 Remove and discard the supernatant. The pellet will be loose so take care not to lose the pellet.
- 1.16 Repeat steps 1.13 – 1.15 once.
- 1.17 Let the pellet air dry (at least 15-20min.) by leaving the cap of the tube open.
- 1.18 Resuspend DNA in 50 to 100 µl of TE buffer (depending on amount of cells used).
- 1.19 Incubate at 55°C for 2 hours to help dissolve the DNA and then store at 4°.

Section 2. Restriction digest and ligation:

- 2.1 **Digest genomic DNA.** Prepare restriction master mix (final reaction volume: 20 µl):

For GepNMDi and UPA vectors

Component	For each sample
10X NEB buffer 3	2.0 μ l
100X BSA (NEB)	0.2 μ l
<i>Apo</i> I (NEB, 10 U/ μ l)	0.5 μ l
PCR water	8.3 μ l

- 2.2 Pipette 11.0 μ l of restriction master mix into each well of a PCR plate
- 2.3 Transfer 9.0 μ l of dissolved gDNA to each well
- 2.4 Incubate 2.5 h at 50°C, then 20 min at 80°C to inactivate the enzyme. Cool to 4°C.
- 2.5 **Prepare splinkerette adaptor.** While the restriction digest is occurring, prepare the splinkerette adaptor.

Component	For each sample
Primer SpAa, 10 μ M:	0.15 μ l
Primer SpBb, 10 μ M:	0.15 μ l
SuRE buffer M (Roche) or Buffer 2 (NEB)	0.05 μ l
PCR water:	0.65 μ l

- 2.6 Incubate Splinkerette mixture for 5 min at 97°C in a heat block. Turn off heat block and let tube cool to room temperature in block to allow annealing of complementary SPLK oligos.
- 2.7 **Ligate adaptor to digested DNA.** Prepare ligation master mix as follows (final reaction volume will be 30 μ l):

Component	For each sample
Splinkerette (from step 2.5)	1.0 μ l
5X DNA Ligation buffer	6.0 μ l
T4-DNA Ligase (400 U/ μ l)	1.0 μ l
PCR water	2.0 μ l

- 2.8 Pipette 10.0 μ l ligation mix directly to each tube of digested (and heat-inactivated) DNA and mix well.
- 2.9 Incubate overnight (12-16h) at 16°C.

Section 3. Purification of Ligated DNA:

- 3.1 Use a PCR purification kit to purify the ligated products away from non-ligated splinkerette adaptors.

Section 4. First Round PCR:

- 4.1 Select primers appropriate for gene trap vector.

Vector Name	5' SPLK Primers	3' SPLK Primers
GepNMDi3	1. Sp0F 2. AdiPCR1R	1. Sp0F 2. Spl3Gep4FI
UpA	1. Sp0F 2. Spl5-UPA-R1	1. Sp0F 2. Spl3-UPA-1F3

4.2 Prepare 1st PCR master mix.

Component	For each sample
PCR water	15.05 µl
10X rxn buffer (-MgCl ₂)	2.5 µl
50mM MgCl ₂	0.875 µl
10mM 4-dNTP mix	0.5 µl
Primer 1 (10 µM)	0.5 µl
Primer 2 (10 µM)	0.5 µl
Taq Polymerase (5 U/µl)	0.1 µl
gDNA	5.0 µl
Final volume	25 µl

4.3 Run 1st PCR program.

94°C for 2min

3 cycles of [94°C for 20 sec, 64°C for 15sec, 72°C for 5 min]

30 cycles of [94°C for 20sec, 58°C for 15sec, 72°C for 3 min]

72°C for 10 min

4°C hold

*Section 5. Second Round PCR:*5.1 Dilute 1st round PCR reactions by adding 50 – 100 µl PCR water to each reaction. Mix well.

5.2 Select primers appropriate for gene trap vector.

Vector Name	5' SPLK Primers	3' SPLK Primers
GepNMDi3	1. Sp1F 2. iPCR4nestF	1. Sp1F 2. Spl3Gep2FII
UpA	1. Sp1F 2. iPCR4nestF	1. Sp1F 2. Spl3Gep2FII

5.3 Prepare PCR master mix.

Component	For each sample
PCR water	19.05 µl
10X rxn buffer (-MgCl ₂)	2.5 µl
50mM MgCl ₂	0.875 µl
10mM dNTP mix	0.5 µl
Primer 1 (10µM)	0.5 µl
Primer 2 (10µM)	0.5 µl
Taq Polymerase (5 U/µl)	0.1 µl
gDNA	1.0 µl
Final volume	25 µl

5.4 Run 2nd PCR program.

94°C for 2min

35 cycles of [94°C for 20 sec, 60°C for 15sec, 72°C for 3 min]

72°C for 5 min

4°C hold

5.5 Analyse 2nd round PCR products on 1.5% agarose gel prior to sequencing.

Section 6. Sequencing:

6.1 Sequence DNA per standard protocols using appropriate sequencing primer(s).

Vector Name	5' Seq Primer	3' Seq Primer
GepNMDi3	iPCR4nestF	Spl3Gep2FII
UpA	iPCR4nestF	Spl3-UPA-F2

*Section 7. Primer Sequences for SPLK PCR:***Common for all clones**

SpAa_ApoI: (for splinkerette adaptor)

CGAAGAGTAACCGTTGCTAGGAGAGACCGTGGCTGAATGAGACTGGTGTGCGACACTAGTGG

SpBb_ApoI: (for splinkerette adaptor)

AATTCCACTAGTGTGCGACACCAGTCTAATTTTTTTTTTCAAAAAA

Sp0F: (for first round PCR)

CGAAGAGTAACCGTTGCTAGGAGAGACC

Sp1F: (for second round PCR)

GTGGCTGAATGAGACTGGTGTGCGAC

Vector-specific primers for PCR and sequencing

AdiPCR1R	TCATCAAGGAAACCCTGGAC
iPCR4nestF	TTGTGGTCTCGCTGTTCCCTT
Spl3Gep4FI	GCAGGCGCATAAAATCAGTC
Spl3Gep2FII	TGACGGGTAGTCAATCACTCAG
Spl5-UPA-R1	CGACGGTATCGATTAGTCCAA
Spl3-UPA-1F3	GGTGTCCGAACTCGTCAGT
Spl3-UPA-F2	TTCCATCTGTTCTGACCTTG

References:

This protocol was modified from the original 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