LIGATION MEDIATED GENOMIC SEQUENCING FOR FOOTPRINTING

Reagents:

1) Linker soln. (prepare ahead of time).

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Longer:  5′-GCGGTGACCCGGGAGATCTGAATTC-3′ (25 mer)
Shorter: 5′-GAATTCAGATC-3′(11 mer)
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The oligonucleotides for the linker must be PAGE purified (11 mer, in particular). For annealing, 6 nmol of longer linker and 2 nmol of shorter are mixed in 40 µl of 1xOPA buffer (50pmol-annealed/µl; for 20 samples) and heated at 95˚C with a water filled heat block. Turn off the heating block and gradually cool down over a period of ~3 hour to room temperature. Store at 4˚C for a week, -20˚C for longer.

2) 10X Vent Polymerase buffer (NEB)

3) 2X Quick Ligase buffer (see p. IV.D.6)

4) Ready-to-Go PCR Beads (Pharmacia)

5) Labeling mix (make fresh, keep on ice)

For 5 reactions:

10-20 pmol end-labeled Primer 3'

1 PCR bead

dH_2O up to 25 µl

-use 5µl for one sample

6) 6% Sequence Gel

7.5 mL 40% Acrylamide/Bisacrylamide (19:1)

10 mL 5X TBE

25g Urea

5mL dH2O (Total 50 mL)

Microwave 15s (**NOT** longer! If solution is hot, make it again).

Cool down to RT (below 4˚C urea begins crystallization).

Add 250 µl of 10% APS and 25 µl of TEMED.

After >1 hour, do pre-run for 30 min.

Procedure:

(a) First strand synthesis reaction

6 µg cleaved DNA
0.3 pmole primer 1(excess primer 1 is not good for LMPCR)
1µl 2.5mM dNTP
1µl 10X Vent Polymerase buffer
1µl Vent Polymerase (**Never** use Taq polymerase)
adjust final volume to 10 µl.

Thermal cycle just **ONE** cycle (never do multi-cycle)
95˚C for 5 min.
55˚C for 5 min.
72˚C for 30 min
4˚C

(b) Ligation reaction

It is not necessary to do phenol-chloroform extraction before ligation.

10 µl sample
100 pmol of double stranded linker (2µl)
12µl 2X Quick ligase buffer
0.5µl 100mM ATP
2 µl T4 DNA ligase (NEB; 800unit)

Incubate at RT for 1-2 hour
Add 2.5 µl 3 M NaOAc and 1µl of glycogen (Roche)
Add 100 µl EtOH, mix and incubate on ice for >20 min
Spin samples 10-15 min. at 4˚C
Wash pellet with 75% EtOH
Resuspend in 20 µl of ddH_2O

(c) PCR and Labeling reaction *(for sequencing or footprinting)*

Mix 20 µl of ligated DNA, 10 pmol of Primer 2 in 5µl of water and one PCR bead.

Denature 95˚C 5 min
25 Cycles of:
denature 30sec. at 95˚C (first denaturing is for 2min.)
hybridize 30 sec. at 60˚C
extend 1 min. at 72˚C
72˚C , 4min
20˚C

Add 5µl of labeling cocktail (containing ^{32}P-endolabeled primer 3), mix
Then:
5 cycles of
95˚C for 2 min.
65-69˚C for 2 min.
72˚C for 3 min.
72˚C, 5min
4˚C
Add 60µl of ddH_2O and 10µl of NaOAc
Phenol/Chloroform/isoamyl alcohol (25:24:1) extraction
Precipitate with 3 volumes of EtOH
Spin, wash with 75% EtOH, resuspend pellet up in 10 µl loading dye (100% Formamide with BPB) and load 3-5 µl on a 6% sequencing gel at 60V.

Comments:

1) All primers should be PAGE purified grade.

Ligation-mediated PCR (LM-PCR) requires **three** gene-specific primers and one set of **linker** oligonucleotides that are annealed.

Gene-specific primers 1, 2 and 3

For human Hsp70 promoter:

Non-coding strand

Primer 1: 5'-AAGACTCTGGAGAGTTCTGA-3'(20mer)
Primer 2: 5'-GGCCTCTGATTGGTCCAAGGAAGGC-3' (25mer)
Primer 3: 5'-GGCCTCTGATTGGTCCAAGGAAGGCTGGG-3' (29mer)

Coding strand

Primer 1: 5'-CCCTGGGCTTTTATAAGTCG-3'(20mer)
Primer 2: 5'-ACGGAGACCCGCCTTTTCCCTTCTG-3' (25mer)
Primer 3: 5'-ACGGAGACCCGCCTTTTCCCTTCTGAG-3' (27mer)

*(see the section "PREPARATION OF THE ^{32}P-LABELED OLIGONUCLEOTIDEPROBE" for end-labeling of Primer 3)

2) The design of the gene-specific primers are critical for the success of the LM-PCR. The followings may be useful for your consideration.
   - The length of the primers are 18-25 mer for primer 1, 20-28 mer for primer 2 and 30-35 mer for primer 3.
   - The G/C content of the primers should increase with each primer. G/C contents of 48% (primer 1), 56% (primer2) and 53-60% (primer 3) have worked well.
   - Primer 3 should not be 50-100 bp away from the region of interest. The DNA sequence could be usually read from just after the Primer 3 up to 200-250 bp.
   - It is ideal to have primer 3 overlap primer 1 by at least half of its sequence.

3) The most important step is the ligation. Ligation efficiency depends on DNA concentration and ATP. Additional ATP may increase efficiency. Primer 1 extention must be done with Vent pol or Pfu pol. Taq or other thermal DNA polymerases may add extra A at 3' and make the double strand DNA cohesive end. Vent Polymerase is much cheaper than Pfu polymerase.

References:

Ausubel et. al. "Ligation-mediated PCR for genomic sequencing and footprinting." Current Protocols in Molecular Biology. Suppl. 20

Mueller, P.Q., and B. Wold. (1989). In vivo footprinting of a muscle specific enhancer by ligation-meditated PCR. Science 246, 780-786.

Carey, M. and Smale, S. T. " Procedure for in vivo foot-printing" Transcriptional Regulation in Eukaryotes (2000) Cold Spring Harbor Laboratory Press. pp355-358