**LIGATION MEDIATED GENOMIC SEQUENCING FOR FOOTPRINTING**

**Reagents:**

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

*Longer:* 5’-GCGGTGACCCGGGAGATCTGAATTC-3’ (25 mer)
*Shorter:* 5’-GAATTCAGATC-3’ (11 mer)

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
- dH₂O 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:**

III.C.24
(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 ddH₂O

(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 \( ^{32} \text{P}-\text{endolabeled primer 3} \)), 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\(_2\)O 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’-GGCCTCTGATTTGGTCCAAGGAAGGC-3’ (25mer)  
Primer 3: 5’-GGCCTCTGATTTGGTCCAAGGAAGGGTG-3’ (29mer)

**Coding strand**
Primer 1: 5’-CCCTGGGCTTTTATAAGTCG-3’ (20mer)  
Primer 2: 5’-ACGGAGACCCGCTTTCCCTTCTG-3’ (25mer)  
Primer 3: 5’-ACGGAGACCCGCTTTCCCTTCTGAG-3’ (27mer)

*(see the section “PREPARATION OF THE $^{32}$P-Labeled Oligonucleotide Probe” 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% (primer 2) 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:**
