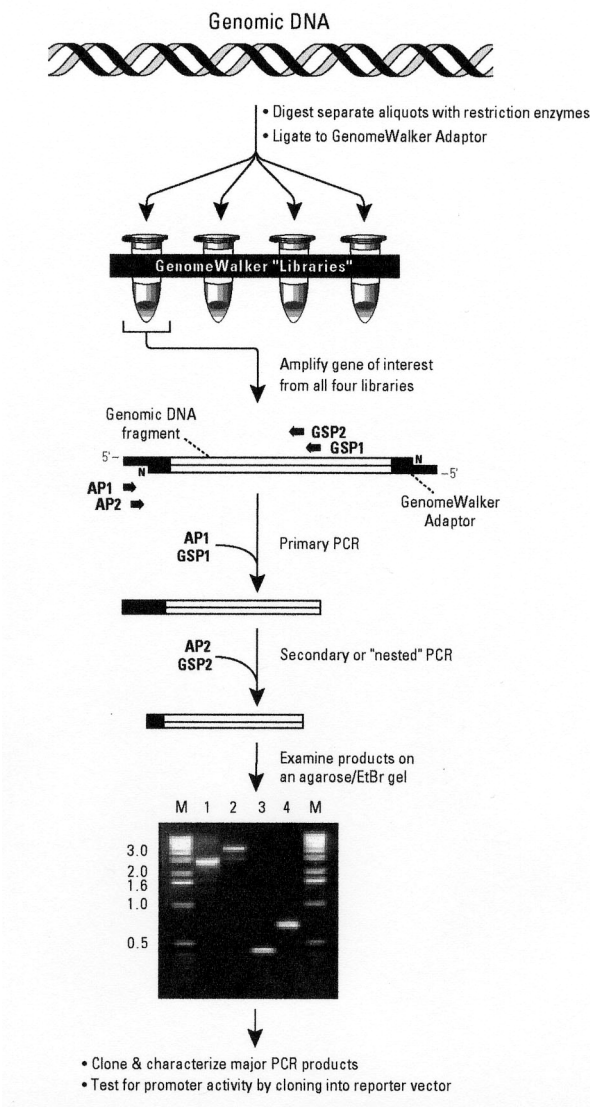
# PCR BASED GENOME WALKING

This protocol was adapted from Siebert et al (An improved PCR method for walking in uncloned genomic DNA , *Nucleic acid research*, 1995, vol 23, N.6, p1087-1088.), and a protocol from the clontech kit (web site: www.clontech.com, Protocol PT3042-1, version PR03300) by Gwyneth Ingram and Karine Coenen.

The protocol involves a nested PCR with a touch down program. This method is very prone to artefacts so you NEED to follow rigorously the requirements for the design of primers and the touch-down PCR program!

## General outline



## Digestion of genomic DNA

1. Cut with a 6bp blunt end cutter. Can either use one enzyme or set up 4 different enzyme reactions to maximize gene coverage. Set up reaction:
   - 2.5 μg of DNA
   - 5 μl of restriction enzyme
   - 10μl 10 x restriction buffer
   - 10 μl 1mgml$^{-1}$ BSA (if not already in 10 x buffer)
   - $dH_2O$ to a final reaction volume of 100μl.

2. Incubate for 5h at the appropriate temperature.

3. Add 100μl 25:24:1 phenol:chloroform: IAA. Vortex and spin for 5' in microfuge. Remove aqueous layer to fresh tube.

4. Add 2.5 volumes ethanol and 0.1 volume 3M NaOAc pH4.5. Precipitate at –20°C overnight or at –80°C for 1 hr.

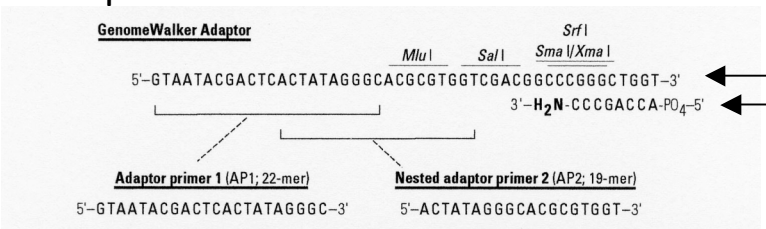
5. Spin in microfuge for 10' at full speed. Wash pellet with 70% ethanol, spin again and drain off supernate.

6. Resuspend pellet in 20μl $dH_2O$.

7. Run 1 μl on a 1% gel, 30V for 5 hours. A good smear should be seen.

## Adapter Ligation

1. To make the adaptor, mix the long and short primers (see below) in the right concentration to get a 25mM final concentration [e.g. 20μl long primer (50mM) + 20μl short (50mM)]. Place at 100°C for 2' and then let cool to room temp.




long adapter primer
short adapter primer

**Figure 3. Structure of the GenomeWalker adaptor and adaptor primers.** The adaptor is ligated to both ends of the genomic DNA fragments to create GenomeWalker libraries. The amine group on the lower strand of the adaptor blocks extension of the 3' end of the adaptor-ligated genomic fragments, and thus prevents formation of an AP1 binding site on the general population of fragments. The design of the adaptor and adaptor primers is critical for the suppression PCR effect (Figure 5). The $T_m$'s of AP1 and AP2 are 59°C and 71°C, determined by nearest neighbor analysis (Freier *et al.*, 1986).

2. Used a **concentrated** T4 DNA ligase (5x), (Biolabs, M0202T) and set up the following reaction:

5 μl of digested DNA
1 μl 10X ligase buffer
1 μl T4 ligase
2.4 μl adaptor (25mM)
0.6 μl dH20

3. Incubate overnight at 16°C

4. Stop the ligase activity by incubating the reactions at 70°C for 5'

5. Add 90 ul TE (10mM Tris pH7.5, 1mM EDTA). This gives you 100μl of a library that can be used for more than a 200 PCR reactions.

## Touch down PCR

Two nested reactions need to be carried out using the AP1 and AP2 primers illustrated on the previous page and two gene specific primers. The gene specific primers need to be designed as follows:

You will need to design two gene-specific primers—one for primary PCR (GSP1) and one for secondary PCR (GSP2). The nested PCR primer should anneal to sequences beyond the 3' end of the primary PCR primer (i.e., upstream of the primary PCR primer when walking upstream and downstream of the primary PCR primer when walking downstream). Whenever possible, the outer and nested primers should not overlap; if overlapping primers must be used, the 3' end of the nested primer should have as much unique sequence as possible.

In general, the gene-specific primers should be derived from sequences as close to the end of the known sequence as possible. For walking upstream from cDNA sequence, the primer should be as close to the 5' end as possible. Ideally, the primers should be derived from the first exon of the gene. If primers are derived from downstream exons, the resulting PCR products are less likely to contain the promoter, particularly if the intervening intron(s) and exon(s) comprise more than a few kb (see Figure 2).

Gene-specific primers should be 26–30 nucleotides in length and have a G/C-content of 40–60%. (Even if the $T_m$'s seem high, do not design primers shorter than 26 bp. At CLONTECH, we typically use 27-mers.) This will ensure that the primers will effectively anneal to the template at the recommended annealing and extension temperature of 67°C. Primers should not be able to fold back and form intramolecular hydrogen bonds, and sequences at the 3' end of your primers should not be able to anneal to the 3' end of the adaptor primers. There should be no more than three G's and C's in the last six positions at the 3' end of the primer.

Five restriction sites have been incorporated into the GenomeWalker Adaptor—*Sal* I (cohesive ends), *Mlu* I (cohesive ends), and overlapping *Srf* I (cohesive ends), *Sma* I (blunt ends), and *Xma* I (cohesive ends) sites. The sites in the Adaptor Primer allow easy insertion of PCR products into commonly used promoter reporter vectors. If you wish to use other restriction sites to clone the resulting GenomeWalker products, suitable sites should also be designed into the 5' end of GSP2 (i.e., the nested gene-specific primer used for secondary PCR.) Alternatively, GenomeWalker products can be cloned into any cloning vector using restriction sites, or into a TA-type cloning vector using the A overhang left by *Tth* and *Taq* DNA polymerases. (See Section VII.B.3 for a discussion of CLONTECH's various promoter-cloning reporter vectors and reporter assay systems.)

1. Set up following reaction using an expand or other enhanced polymerase

| | |
|---|---|
| 1 μl | adapter ligated library |
| 5 μl | 10 X PCR buffer |
| 4 μl | 25 mM $MgCl_2$ |

| | |
|---|---|
| 1 μl | 10 mM dNTP's |
| 1 μl | AP1 Primer (10μM) |
| 1 μl | Gene specific primer 1 (10μM) |
| 0.5 μl | DNA Polymerase |
| 35.5 μl | $dH_2O$ |
| 50 μl | TOTAL |

2. Cycle as follows:

| | |
|---|---|
| 94°C (25s), 72°C (3') | X 7 |
| 94°C (25s), 67°C (3') | X 32 |
| 67°C (7') | X 1 |
| cool to 4°C. | |

3. Analyze 8μl of the reaction on a 1.5% agarose gel. You should observe banding patterns however there may be some smearing.

4. Dilute 1μl of each primary PCR into 49 μl $dH_2O$.

5. Set up nested reaction mix:

| | |
|---|---|
| 1 μl | diluted primary PCR reaction |
| 5 μl | 10 X PCR buffer |
| 4 μl | 25 mM $MgCl_2$ |
| 1 μl | 10 mM dNTP's |
| 1 μl | AP2 Primer (10μM) |
| 1 μl | Gene specific primer 2 (10μM) |
| 0.5 μl | DNA Polymerase |
| 35.5 μl | $dH_2O$ |
| 50 μl | TOTAL |

6. Cycle as follows:

| | |
|---|---|
| 94°C (25s), 72°C (3') | X 5 |
| 94°C (25s), 67°C (3') | X 20 |
| 67°C (7') | X 1 |
| cool to 4°C. | |

7. Analyze 5μl of the reaction on a 1.5% agarose gel. You should observe distinct banding patterns. The remainder of the PCR reaction can then be used to clone and sequence the band of interest.