INVERSE PCR (Susie)

1. Digest approximately 2 \(\mu\)g of genomic DNA with the appropriate enzyme in a 50 \(\mu\)l reaction. i.e.:
   - DNA: 2 \(\mu\)g
   - 10x buffer: 5 \(\mu\)l
   - spermidine (100mM): 1.5 \(\mu\)l
   - restriction enzyme (10 U/\(\mu\)l): 2 \(\mu\)l
   - d\(H_2O\): to 50 \(\mu\)l

2. Clean the digest using a Wizard DNA Clean-up System kit (or other similar method – final volume should be about 50 \(\mu\)l)

3. Self ligate 12.5 \(\mu\)l of the cleaned DNA in a 100 \(\mu\)l reaction. i.e.:
   - DNA: 12.5 \(\mu\)l
   - 10 x T4 DNA ligase buffer: 10 \(\mu\)l
   - T4 DNA ligase: 1 unit
   - d\(H_2O\): to 100 \(\mu\)l

4. Incubate at 12\(^\circ\)C overnight.

5. The PCR is based on the Boehringer Mannheim Expand Long Template PCR System protocol. Set up two reactions, one with about 10 \(\mu\)l of ligated DNA and the other with about 20 \(\mu\)l of ligated DNA. A hot-start method is used, so use two 25 \(\mu\)l mixes for each 50 \(\mu\)l reaction, as follows:

   **Mix 1**
   - ligated DNA (see step 3): 10/20 \(\mu\)l
   - dNTPs (25 mM): 1.25 \(\mu\)l
   - each primer (10 \(\mu\)M): 1.5 \(\mu\)l
   - d\(H_2O\): to 25 \(\mu\)l

   **Mix 2**
10x buffer 3 (Expand Long Template System kit) 5 µl
Expand enzyme mix (DNA polymerases) 0.75 µl
dH₂O 19.25 µl

6. Overlay Mix 1 with about 30 µl paraffin oil, then heat the tubes to 94°C, in the first step of the PCR. Once the tubes are at 94°C, add Mix 2, beneath the oil. Then run the PCR:

- 35 rounds of 94°C 10 secs
- 55°C 30 secs
- 68°C 2 minutes
- then 68°C 10 minutes

7. A nested reaction can then be carried out, either using 1 µl of the first reaction as template, or by running the reaction on a gel, gel-extracting the band of interest and then using this as template. I found the second method gave a clearer, stronger band when the nested PCR products were subsequently run on a gel.