CLONING INVERTED REPEATS IN HIGH THROUGHPUT

This protocol is calculated for cloning one 96 well plate

1. design primers: as standard we include a EcoRI restriction site on the 5' primer and XbaI on the 3' primer; the primers should to amplify 300-400 bp of coding region (either on genomic DNA or cDNA; see separate protocol for 1st strand cDNA preparation and genomic DNA prep, respectively);
   we designed all primers using Primer3:

   Product Size min 300 bp, opt 350 bp, max 400 bp; Primer Size min 20 bp, max 36 bp; Primer TM min 66°C, opt 68°C, max 70°C, max difference 100°C; CG content min 25 %, max 75 %; Max Self Complementarity 5; Max 3' Self Complementarity 2 (the values were set less stringent if no primers could be found at the first try)

   the cloning strategy is first to digest the PCR fragment first with EcoRI, then ligate with itself to obtain inverted repeats, secondly to digest the flanks of the inverted repeat with XbaI, and finally paste the fragment into a vector also cut with XbaI;

2. PCR (see PCR protocol):

   PCR program
   38 cycles of 94°C for 30 sec, 58° for 30 sec and 72° for 45 sec

   100 µl total reaction
   0.4 µM each primer
   2 mM MgCl2
   200 µM dNTPs (each)
   0.5 µl Taq (e.g. FIREPol, Solis Biodyne)
   template: 1µl genomic DNA or 3 µl cDNA

3. control gel of PCRs
   load 4 µl of the 100 µl PCR reaction on a 2% agarose-gel
4. SIGMA-purify (see SigmaSpin protocol) 50 µl PCR (final volume ~ 50 µl)
5. set up first digest with e.g. EcoRI:
   after SigmaSpin purification (eluate ca. 50 µl): directly add only buffer & enzyme to the purified DNA:

   ca. 50 µl DNA
   3 µl EcoRI
   5 µl buffer H } added as premix

mix by gently pipetting up and down

6. incubate for 3 – 4 hrs
7. heat-inactivate digest (65°C / 20min) and SIGMA-purify digest (final volume of eluate ~ 57 – 60 µl)
8. set up ligation:
   directly add only buffer & ligase (we used T4 DNA ligase from Roche) to purified PCR fragment (see 5.)
9. incubate O/N 16°C
10. heat-inactivate ligation (65°C / 10min) and SIGMA-purify ligation (final volume of eluate ~ 65 µl)
11. set up second digest (again 3– 4 hrs incubation) with e.g. XbaI (see 5.)
12. load the digest on one a 2% agarose-gel for gelextraction
13. DNA extraction with NucleoSpin Gel Extraction Kit (see seperate protocol)
14. digest cloning vector (can be done in parallel with 11.)

    set up 4 digests with each:

    2 µg vector (e.g. pUAS or pMF3*)
    3 µl encyme
    in total 50 µl

    after 3 - 4 hrs: directly add 1.5 µl CIP (Alcaline Phosphatase from Roche) to each digest, keep at 37°C for 30', then add another 1.5 µl CIP and incubate for 30' longer
    *pMF3 is a 10XUAS transformation P-element (see vector map and sequence)

15. gelpurify vector (e.g. 0,7% agarose gel) with QIAQUICK (one QIAQUICK column per digest is enough):
    elute with <u>100</u> µl EB from one column => from 4 digests you get 400 µl eluate, enough for 96 ligations
16. set up ligations in fresh micro well plate:

    4 µl vector (from 14.)
    13 µl inverted repeat fragment (from 12.)
    2 µl ligase buffer
    1 µl ligase } added as premix

    mix by gently pipetting up and down

17. incubate O/N at 16°C

Bacteria transformation

- to each well of a 96 micro well plate (already containing the 20 µl ligation mixture) add 40 µl chemo-competent SURE cells (Stratagene) and keep 30' on ice
- heat shock in the PCR-machine 1'30" at 42°C''
- recover on pre-chilled aluminium block (that can fit the 96 micro well plate) on ice for 5'
- transfer transformed bacteria into prepared 2.2 ml 96 deep well block (square wells with conical base) containing 900 µl 2XTY (LB is also fine) medium (pre-warmed to 37°C)
- seal plate with adhesive film and incubate at 37°C for 30'
- centrifuge block: 4000 rpm, 5 min
- pour off medium quickly (pellet is very loose!) and briefly dry block surface (block still holding upside down) with paper towel
- add 50 µl 2XTY / well, seal plate, resuspend pellet by vortexing, plate out with the use of glass beads (e.g. 4 mm glass beads from Scherf Präzision)

Qiagen Turbo Miniprep 96

equipment needed:
- QIAvac unit or similar
- Centrifuge capable of spinning 96 well plates (we used a Heraeus 3SR)
- plastics: a 6 ml 48 deep well block (pyramidal bottom, e.g. from ABgene
- ultra rigid 96 micro well plates, e.g. Thermo-Fast 96 Skirted – Ultra Rigid Plates from ABgene
- normal 96 micro well plates

- inoculate SURE bacteria colonies in 3,5 ml LB medium per well in a 6 ml 48 deep well block (pyramidal bottom, from ABgene)
- puncture adhesive film sealing, seal block and grow bacteria for 15 - 16 hrs at 37°C in a shaker
- centrifuge block: 3000 rpm, 5';
- poor off SN, dry surface with paper towel (plates can be frozen away now for some days at least)
- add 250 µl (always per well) P1, seal plate;
  vortex until pellet is completely resuspended
- add 250 µl P2, seal plate;
  shake with hand (not vortexing!) until suspension becomes clear and viscous;

stand for 5 – 7 min (if necessary shake again and wait longer; efficient lysis of bacteria is crucial!);

remove sealing, dry surface of block with towel

- add 350 µl N3, seal plate;

  shake immediately with hand thoroughly (not vortexing!)
- place blue column plate onto plate holder inside QIAvac;
- place filter plate onto column plate;
- load with big multipette 450 µl from block onto filter plate;
- suck filtrate into column plate (< 500 mbar);
- load rest onto filter plate and suck remaining filtrate into column plate;

  if some filter plate wells get clogged, open them by gently poking into the precipitate (don't make holes in the filter paper!) in a well with a yellow tip
- switch off vacuum, place waste tray into QIAvac and blue column plate on top
- discard filter plate
- suck liquid from column plate into waste tray;
- wash column plate with 2 X 900 µl PE

  (pipette PE, wait 5', suck through, pipette PE, wait 5', suck through)
- let vacuum switched on for 10 min
- seal column plate and centrifuge remaining PE into waste 96 micro well plate (4800rpm, 5')
- dry blue column plate at 65°C for 10 min
- put column plate onto fresh 96 micro well plate (use ultra rigid plates, e.g. Thermo-Fast 96 Skirted - Ultra Rigid Plates from ABgene since normal micro well plates get compressed), add 80 µl EB, stand 2 min and elute DNA by centrifugation (4800 rpm, 5')
- control digest: 4 µl DNA in total 20 µl with XbaI;
- load on agarose gel (500 ml, e.g. 2%, 4 combs for 96 Minis)

Important Notes:

control digest:

don't expect EcoRI (the site that sits in the middle of your inverted repeat) to work properly, the reason must be the weird DNA structure; if you obtain the expected band and an equally strong, weaker or even absent band of half the expected size, the clone is o.k. (the lower band is just due to folded back hairpins after digestion with XbaI; also the digest often is not complete); we used DNA that looked like just described to make transgenic flies and in 85% of cases the insertions that I get are fine by PCR-check and sequence; for PCR-checks you have to use the sense (EcoRI) primer and a primer that sits either in the hsp promoter or the SV40 3' UTR of the UAS vector ; PCR of the entire inverted repeat doesn't work;
if all your clones look weird upon control digest (just undigested vector, weird bands, etc.) you either grew the culture too long or the sequence is simply too recombinogenic in bacteria

(you cannot predict which sequences will work and which won't, but the difficulty to clone inverted repeats is dependent on the length of the sequence);
in latter case, it is best to choose another target region;
also don't forget that XbaI is methylation sensitive (so the XbaI control digest might not work), therefore check your primer sequence before and if necessary introduce one additional nucleotide to destroy the methylation site;