Recombineering protocol #1

Recombineering using the modified DH10B strain DY380

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Overview

1. Design primers with 50 bp homology suited for either targeting (insertion of a selectable marker into a specific site) or gap repair (retrieving a piece of DNA into a linear piece of vector backbone).
2. Run PCR and digest residual plasmid template away with *Dpn*I (1-2 µl in a standard 25 µl PCR reaction, @37°C for 1 h). *Dpn*I works fine in PCR buffers. Gel purify the PCR product and elute in ddH_2O. If concentration is low, EtOH precipitate and dissolve in small volume of ddH_2O. You need 2 x 100-300 ng rather concentrated PCR product per experiment.
3. Alternatively, design primers to amplify "mini-arms" of 250-500 bp length, and clone these arms into an appropiate vector using conventional cloning techniques. The vector is prepared by digestion/linearization and gel purification of the desired band. Background will be higher than with *Dpn*I digested PCR fragments, but the efficiency is very high when the arms are longer.
4. Transform your target plasmid (or BAC) into electrocompetent DY380 cells, see below. Alternatively, co-transform target plasmid and PCR product (see note below).
5. From a single isolated colony, inoculate a 5 ml o/n culture with appropriate selection
6. Perform the heat shock induction, followed by transformation (the recombineering experiment).

Transformation (electroporation) of DY380

1. Make a 5 ml o/n culture either from the DY380 freeze stock or from a colony containing a target plasmid. **IMPORTANT**: Keep the cells @ 32°C or lower. The DY380 strain is resistant to tetracycline (12.5 µg/ml), but it is not necessary to include selection in this step – unless you are afraid of contamination. If a target plasmid/BAC is already in the strain, use the appropriate antibiotic in order not to lose the plasmid/BAC *(you don't have to double select using both tet and the other marker – the plasmid marker selection is enough).*
2. Dilute the o/n culture 1:50, i.e. transfer 500 µl into an autoclaved 50 ml Erlenmeyer baffled flask with 25 ml LB. Incubate for 3-5 hours with appropriate antibiotic selection in a 32°C shaking waterbath until the density reaches an OD_{600} of 0.6. Remember to save 1 ml LB before adding the 500 µl o/n culture. Use this as a reference when measuring the OD. Put a bottle of 10% glycerol or ddH_2O in an ice/waterbath slurry. *(If the competent cells are to be used right away, use ddH_2O. If the competent cells are going to be stored @ -70°C for later use, you must use 10%*

glycerol. In the following ddH_2O is used, but this should be substituted with 10% glycerol if appropriate).

3. When the OD_{600} is 0.6 the flasks containing the bacteria are cooled down in the ice/waterbath slurry for a minute or two and subsequently transferred into pre-cooled 15 ml Falcon tubes.
4. Spin down the bacteria in a cold (0°C) centrifuge for 5 min @ 5000 RPM (standard Beckman or Eppendorf centrifuge)
5. Pour off <u>all</u> supernatant and briefly invert tube on a paper towel, and add 1 ml ice-cold ddH_2O while keeping the tube in the icewater. Resuspend the pellet in the ddH_2O by gently shaking the tube in the ice/waterbath *(Gently move the tubes around in circles while keeping them in the ice/water slurry. This can take a while the first time, around 5 minutes. When the cells are prepared for electroporation after heat shock, see later, you might notice that the un-induced half is more difficult to get into suspension than the induced one).* When resuspended, fill up to 10 ml with ice cold ddH_2O, invert a couple of times, and spin again for 5 minutes.
6. Pour off supernatant, resuspend as in step 5 *(this time resuspension is much faster)*, and spin once more.
7. Gently remove ALL supernatant by inverting the tube on a paper towel or shake out the final bit – be careful so you don't lose the pellet. Store the competent cells on ice. Gently resuspend the pellet as before, but in the residual small amount of ddH_2O left in the tube *(volume should be around 50 µl).*
8. Transfer 25 µl of the freshly made electrocompetent cells to a pre-cooled eppendorf tube and mix with the DNA to be transformed (1-5 µl DNA). Transfer to a pre-cooled 0.1 cm cuvette. To put in a target plasmid for subsequent recombination, use 1-10 ng supercoiled plasmid. To put in a BAC, use 1-5 µg relatively freshly prepared BAC DNA *(DNA made same day works best, but even DNA stored a couple of months in your –20°C freezer will work).*
9. Transform by electroporation. Transfer the bacteria to a tube with 1 ml LB medium. Incubate @ 32°C in a shaking waterbath for 1 h.
10. Plate the transformed bacteria on selective medium (see note below), *e.g.* 1 µl, 10 µl, 100 µl, and the rest *(transfer to an eppendorf tube, spin down @ full speed for 10 seconds, and remove most of the supernatant. The bacteria will become "hairy" or "fluffy" after the 1 hour incubation. This is quite normal and doesn't influence the outcome of the experiment. When plating "the rest", it might be an advantage to pipet up and down BEFORE the 10 sec spin. The cells don't become "fluffy" when heat-shocked, see below).*
11. Incubate @ 32°C for 18-24 hours.

Heat shock induction of the λ Red recombination proteins – addition to the transformation protocol

1. When the bacteria containing the target plasmid/BAC have reached an OD_{600} of 0.6, transfer 10 ml to an autoclaved 50 ml Erlenmeyer flask, and put the rest (10-12 ml) back @ 32°C. The 10 ml are incubated in a shaking waterbath @ 42°C for *exactly* 15 min.
2. Cool down the bacteria by gently shaking the flask in the ice/waterbath for a minute or two. Pour the bacteria into 15 ml Falcon pre-cooled tubes (one for the bacteria grown @ 32°C and one for the heat-shocked bacteria incubated @ 42°C) and place in the ice/waterbath slurry. Go to step 4 in the transformation protocol above.
3. Transformation of the bacteria grown @ 32°C only (*i.e.* no heat shock) is the control experiment. No or very few colonies should appear on this plate. When using longer arms and preparation of the vector by digestion/gel preparation, instead of PCR + *Dpn*I digest, the background will be higher – still the ratio between the heat-shocked and the control plate should be significant.
4. Check a number of colonies (miniprep followed by digestion or PCR). For targeting experiments using plasmids, the bacteria might contain both un-recombined and recombined plasmid. You can purify correct clones by re-transforming the purified plasmid into e.g. DH10B and select for the antibiotic resistance present only in the targeted plasmid *(often this would be kanamycin).* Alternatively *(works with 100% efficiency)*, linearize the miniprep DNA, dilute, selfligate, and transform bacteria – select for the antibiotic resistance present only in the targeted plasmid *(this way, if the targeted and un-targeted plasmids were entangled, the targeted plasmid is released and can be purified after being re-transformed).*

Notes

(1) Antibiotics and selection: Plate on LB plates with appropriate selection: For gap repair *(also known as "retrieval")* select for the resistance present in the backbone of the PCR product/retrieval vector and NOT the resistance of the target plasmid (MUST be different). For targeting, co-select for the resistance genes present on BOTH the PCR product/targeting vector and the target plasmid. We use the following concentrations:

- BACs
  - chloramphenicol (cm): 12.5 μg/ml
  - kanamycin (kan): 25 μg/ml
  - ampicillin (amp): 50 μg/ml
  - tetracyclin (tet): 12.5 μg/ml
- Plasmids
  - cm: 25 μg/ml
  - amp: 100 μg/ml,
  - kan: 50 μg/ml

(2) LB media: For 1 liter: 10 g Tryptone, 5 g Yeast extract, 5 g NaCl, ddH_2O up to 1 liter. Autoclave.

(3) Co-transformation: Instead of first introducing the target plasmid into DY380 followed by heat-shock and transformation with a PCR product/targeting cassette, the target plasmid and the PCR/targeting cassette can be co-transformed into +/- heat-shocked and electro-competent DY380. The efficiency will be lower, but with this strategy you don't have to first introduce the target plasmid, and you often don't have to purify by re-transformation. With BAC modifications/retrieval, however, you should always put the BAC in the strain first, followed by transformation with your PCR product/retrieval vector/targeting cassette *(the efficiency of linear DNA + BAC co-transformation is simply too low)*. Using 50 bp homology arms and PCR products for targeting, I suggest that the target plasmid is introduced into the strain first. However, using the longer homology arms, co-transformation can be very efficient.

(4) Oligos for recombineering experiments: Use your favorite oligo-provider for standard primers for PCR, say up to 30-40 bp. However, when using longer primers, up to 100 bp, choosing a good oligo-provider can be crucial. We are pretty happy about the oligo quality from Invitrogen, so we always use them for our long oligos. No special purification, just desalting.