

BAC Recombineering using the modified DH10B strain SW102 and a *galK* positive/counterselection cassette

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Introduction

This protocol explains in detail how to use the *galK* positive and counterselection scheme to make essentially any BAC modification (e.g. point mutations, deletions, and insertions). The modified BAC can then be used directly for making BAC transgenic mice by pro-nuclear microinjection, or a fragment containing the modification can be retrieved using gap repair and turned into a gene targeting vector for use in mouse ES cells. The BAC modifications are done using a modified bacterial strain, SW102. This strain is derived from DY380 and therefore contains the λ prophage recombineering system. Furthermore, the galactose operon has been modified so it is fully functional, except the galactokinase gene (*galK*) has been deleted. Importantly, the *galK* function can be added *in trans*, and thereby the ability to grow on galactose as carbon source is restored. The *galK* selection scheme is a two-step system: First the *galK* cassette, containing homology to a specified position in a BAC, is inserted, by homologous recombination, into the BAC. The recombinant bacteria will now be able to grow on minimal media with galactose as the only carbon source, so the first step is positive selection. Second, the *galK* cassette is substituted by an oligo (double- or singlestranded), a PCR product, or a cloned fragment with homology flanking the cassette. This is achieved by selecting against the *galK* cassette by resistance to 2-deoxy-galactose (DOG) on minimal plates with glycerol as the carbon source. DOG is harmless, unless phosphorylated by functional *galK*. Phosphorylation by *galK* turns DOG into 2-deoxy-galactose-1-phosphate, a non-metabolizable and therefore toxic intermediate. From the resulting DOG-resistant colonies some will be background colonies, where the bacteria have lost the *galK* cassette by a deletion, and the rest will be truly recombinant clones. Even with as low as 33 bp homology on both sides of the oligo we have still observed an efficiency of 50% correct clones, and with 50 bp homology arms we routinely observe 60-80% efficiency. Longer homology arms might improve the efficiency even more. Chloramphenicol selection is used throughout, in order to maintain the BAC.

Protocol

1. Design *galK* primers with 50 bp homology to an area flanking the desired site to be modified. The 3' end of these primers bind to the *galK* cassette. For example, if you want to make a single bp substitution, your homology arms should be the 50 bp on either side of the single bp. This will result in a deletion of that basepair in the first step. If you want to make a small or a

large deletion, design the *galK* primers so the deletion is made already in the first step. The primers should look as follows:

Forward:

5'-----50bp_homology-----CCTGTTGACAATTAATCATCGGCA-3'

Reverse:

5'----50bp_homology_compl._strand----TCAGCACTGTCCTGCTCCTT-3'

2. Design other primers/oligos depending on what the desired modification is. If point mutation, design two 100 bp complementary oligos with the modified basepair(s) in the middle. The remaining bases on either side are homology arms. If a deletion is made with the *galK* cassette in the first step, design two complementary oligos to substitute for *galK*. *This will result in a "seam-less" deletion, leaving nothing but the desired change behind.*
3. Choose a BAC for your purpose. If you need a 129 BAC for subsequent construction of a targeting vector, screen a 129 library like the CITB library from Invitrogen. Order the clones from Invitrogen. End sequence the positive clones to find one that contains the sequence needed. If a C57BL/6 BAC is needed, identify a BAC using a genome browser (like <http://genome.UCSC.edu/>), and order the BAC from Invitrogen or CHORI. Before proceeding, make sure the BAC is 100% correct; PCR analysis and restriction enzymatic analysis (fingerprinting). We have had good success with *SpeI* digests – it cuts frequently enough to give many bands, and infrequently enough so that the band sizes are informative. *It's worthwhile spending some time on this BAC characterization – if you don't, you might regret it later...*
4. Transform the characterized BAC into electrocompetent SW102 cells (see recombineering protocol #1). Recover for 1 hour at 32°C, and plate on LB plates with 12.5 µg/ml chloramphenicol.
5. PCR amplify the *galK* cassette using the primers designed in step 1 and a proof-reading Taq-mix (we use Expand High Fidelity from Roche). Use 1-2 ng template (the *pgalK* plasmid). 94°C 15 sec., 60°C 30 sec., 72°C 1 min., for 30 cycles. Add 1-2 µl *DpnI* per 25 µl reaction, mix, and incubate at 37°C for 1 hour. *This step serves to remove any plasmid template; plasmid is methylated, PCR products are not.* Gel-purify the *DpnI*-digested PCR product, preferably overnight at low voltage. From a strong PCR band, purified, and eluted in 50 µl ddH₂O, we use 2.5 µl for a transformation (approx. 10-30 ng).
6. Inoculate an overnight culture of SW102 cells containing the BAC. 5 ml LB + chloramphenicol. Incubate at 32°C.
7. Next day, turn on two shaking waterbaths: One at 32°C, the other at 42°C. Make an ice/water slurry and put a 50 ml tube of ddH₂O in there to make sure it's ice-cold (see later). Dilute 500 µl of the overnight SW102 culture

- containing the target BAC in 25 ml LB with chloramphenicol (12.5 μ g/ml) in a 50 ml baffled conical flask and incubate at 32°C in a shaking waterbath to an OD₆₀₀ of approx. 0.6 (0.55-0.6). This usually takes 3-4 hours.
8. Transfer 10 ml to another baffled 50 ml conical flask and heat-shock at 42°C for exactly 15 min. in a shaking waterbath. The remaining culture is left at 32°C as the un-induced control.
 9. After 15 min., the two samples, induced and un-induced, are briefly cooled in an ice/waterbath slurry and then transferred to two 15 ml Falcon tubes and pelleted using 5000 RPM at 0°C for 5 min. It's important to keep the bacteria as close to 0°C as possible in order to get good competent cells.
 10. Pour off all of the supernatant and resuspend the pellet in 1 ml ice-cold ddH₂O by gently swirling the tubes in the ice/waterbath slurry. No pipetting. This step may take a while. When resuspended, add another 9 ml ice-cold ddH₂O and pellet the samples again.
 11. Repeat step 10.
 12. After the second washing and centrifugation step, all supernatant must be removed by inverting the tubes on a paper towel, and the pellet (approximately 50 μ l each) is kept on ice until electroporated with PCR product.
 13. Transform the now electrocompetent SW102 cells. We use 25 μ l cells for each electroporation in a 0.1 cm cuvette (BioRad) at 25 μ F, 1.75 kV, and 200 ohms. After electroporation of the PCR product, the bacteria are recovered in 1 ml LB (15 ml Falcon tube) for 1 hour in a 32°C shaking waterbath.
 14. After the recovery period the bacteria are washed twice in 1xM9 salts (see appendix A) as follows: 1 ml culture is pelleted in an eppendorf tube at 13,200 RPM for 15 sec. and the supernatant removed with a pipette. The pellet is resuspended in 1 ml 1xM9 salts, and pelleted again. This washing step is repeated once more. After the second wash, the supernatant is removed and the pellet is resuspended in 1 ml 1xM9 salts before plating serial dilutions in 1xM9 (100 μ l, 100 μ l of a 1:10 dilution, and 100 μ l 1:100) onto M63 minimal media plates (see appendix A) with galactose, leucine, biotin, and chloramphenicol. *Washing in M9 salts is necessary to remove any rich media from the bacteria prior to selection on minimal media.* The uninduced samples routinely have a higher degree of lysis/bacterial death after electroporation and you will lose some bacteria, so the uninduced sample is diluted in 0.25 – 0.75 ml 1xM9 salts in the final step to make up for the difference. Plate 100 μ l of the uninduced sample as a control.
 15. Incubate 3 days at 32°C in a cabinet-type incubator.
 16. Streak a few colonies onto MacConkey + galactose + chloramphenicol indicator plates. Streak to obtain single colonies (see appendix B). *The colonies appearing after the 3 days of incubation should be Gal+, but in order to get rid of any Gal- contaminants (hitch-hikers), it is important to obtain single, bright red colonies before proceeding to the second step. Gal- colonies will be white/colorless and the Gal+ bacteria will be bright*

- red/pink due to a pH change resulting from fermented galactose after an overnight incubation at 32°C.*
17. Pick a single, bright red (Gal+) colony and inoculate a 5 ml LB + chloramphenicol overnight culture. Incubate at 32°C. There is normally no need to further characterize the clones after the first step.
 18. Repeat steps 7 through 12 above to obtain electrocompetent SW102 cells (now ready for a *galK* <> mutation substitution). If you are going to transform a double-stranded DNA oligo, the two complementary oligos can be annealed in vitro: Mix 10 µg of each oligo in a volume of 100 µl 1x PCR buffer. Boil for 5 min. Let cool slowly to room temp (30 min.). Add 10 µl 3 M NaAc and 250 µl EtOH. Precipitate, wash once in 70% EtOH, and resuspend the final, air-dried, pellet in 100 µl ddH₂O (final conc. of 200 ng/µl). Use 1 µl per transformation.
 19. Transform the bacteria (25 µl of heat-shocked and 25 µl of uninduced control) with 200 ng double-stranded oligo, a PCR product, or anything containing a mutation and with homology to the area flanking the *galK* cassette. Recover in 10 ml LB in a 50 ml baffled conical flask by incubating in a 32°C shaking waterbath for 4.5 hours. *This long recovery period serves to obtain bacteria, by "dilution", that only contains the desired recombinated BAC, and thus have lost any BAC still containing the galK cassette.*
 20. As in step 14, pellet 1 ml culture and wash twice in 1xM9 salts, and resuspend in 1 ml 1xM9 salts after the second wash before plating serial dilutions (100 µl, 100 µl of a 1:10 dilution, 100 µl 1:100, and 100 µl 1:1000) on M63 minimal media plates with glycerol, leucine, biotin, 2-deoxy-galactose (DOG), and chloramphenicol.
 21. Incubate at 32°C for three days.
 22. The number of colonies may or may not be significantly different when comparing plates from uninduced and induced bacteria (range between 1:1 – 1:100). In either case, you will still be able to find true recombinants with a high frequency. Analyze, say, 10-12 colonies by *SpeI* digestion of BAC miniprep DNA (see appendix C). Include a *SpeI* digest of the parent BAC as a control. Clones with a digestion pattern like the parent are likely to have undergone the desired mutation. Background clones (DOG resistant without the desired mutation) will have obvious deletions, and should not be analyzed further. The clones with correct digestion pattern should be analyzed by PCR and sequencing of the mutated region. *If the pattern is identical to the parental digestion pattern, it means that the bacteria most likely became DOG resistant due to the desired homologous recombination event. Alternatively, any deletion that contains the region with the galK cassette, but excludes the chloramphenicol region, will also be selected in the counterselection procedure. With a high frequency of homologous recombination as in the SW102 strain, the background is not likely to be a problem. In the unlikely event that too high a background is observed, try to increase the length of the homology arms, to increase the frequency of homologous recombination.*

Appendix A. Media (from Current Protocols in Molecular Biology)

M9 medium (1 liter)

1X 6 g Na₂HPO₄
 3 g KH₂PO₄
 1 g NH₄Cl
 0.5 g NaCl

AUTOCLAVE

M63 minimal plates

1L 5X M63 10 g (NH₄)₂SO₄
 68 g KH₂PO₄
 2.5 mg FeSO₄·7H₂O
 adjust to pH 7 with KOH

AUTOCLAVE

Other 0.2 mg/ml d-biotin (sterile filtered) (1:5000)
 20% galactose (autoclaved) (1:100)
 20% 2-deoxy-galactose (autoclaved) (1:100)
 20% glycerol (autoclaved) (1:100)
 10 mg/ml L-leucine (1%, heated, then cooled down and
 sterile filtered)
 25 mg/ml Chloramphenicol in EtOH (1:2000)
 1 M MgSO₄·7H₂O (1:1000)

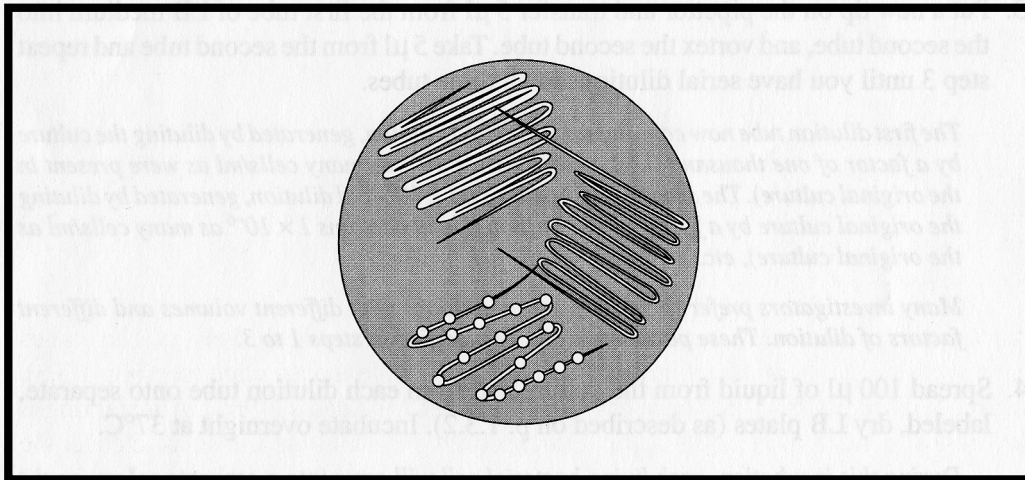
Autoclave 15 g agar in 800 ml H₂O in a 2 liter flask. Let cool down a little. Add 200 ml autoclaved 5X M63 medium and 1 ml 1 M MgSO₄·7H₂O. Adjust volume to 1 liter with H₂O if necessary. Let cool down to 50°C (“touchable hot”). Add 10 ml carbon source (final conc. 0.2%), 5 ml biotin (1 mg), 4.5 ml leucine (45 mg), and 500 µl Chloramphenicol (final conc. 12.5 µg/ml). Pour the plates, 25-40 plates per liter.

MacConkey indicator plates

Prepare MacConkey agar plus galactose according to manufacturer’s instructions. After autoclaving and cooling to 50°C, to one liter add 500 µl Chloramphenicol (final conc. 12.5 µg/ml), and pour the plates, 25-40 plates per liter.

Appendix B. Streaking for single colonies.

Refer to the figure below (from Current Protocols in Molecular Biology). With a sterile toothpick, streak a single colony on a plate, in a forward-and-back motion a couple of times. With a new toothpick, streak in a pattern perpendicular to the first pattern, starting by streaking through the first pattern. Repeat at least once more. This will result in a dilution of the bacterial density, and you should obtain single colonies.



Appendix C. BAC minipreps.

For BAC minipreps (1-1.5 μ g) we use the following protocol: 5 ml overnight LB culture with chloramphenicol (15 ml Falcon tube) is pelleted for 5 min. at 5,000 RPM, the supernatant removed, and the pellet dissolved in 250 μ l buffer P1 (miniprep kit, Qiagen) and transferred to an eppendorf tube. 250 μ l P2 buffer is added, followed by mixing by inversion and incubation for <5 min. at room temperature. Add 250 μ l N3 buffer, followed by mixing and incubation on ice for 5 min. The supernatant is cleared by two rounds of centrifugation at 13,200 RPM for 5 min. in a tabletop centrifuge. Each time the supernatant is transferred to a new tube. DNA is precipitated by adding 750 μ l isopropanol, mixing and incubating on ice for 10 min., and centrifugation for 10 min. at 13,200 RPM. The pellet is washed once in 70% ethanol and the airdried pellet is dissolved in 50 μ l TE. 40 μ l (approximately 1 μ g) can be used for restriction analysis in a 50 μ l reaction, and 1 μ l can be used as template for PCR analysis or for transformation of electrocompetent bacteria.