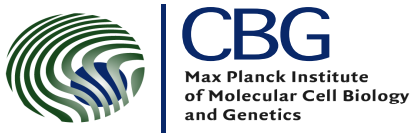
Introduction of mutations/deletions using a counter-selection system (Counter-Selection BAC Modification)



For additional notes and tips refer to BAC modification

Primer Design

There are two counter-selection cassettes in the lab currently. Both use the rpsL gene that confers streptomycin sensitivity. One has attached the neo gene (kanamycin resistance) and one has the amp gene (ampicillin resistance)

The annealing sequence for these cassettes to be placed at the 3' end of the primers are below:

rpsL-neo

Forward: GGCCTGGTGATGATGGCGGGATCG

Reverse: TCAGAAGAACTCGTCAAGAAGGCG

psL-amp

Forward: GGCCTGGTGATGATGGCGGGATCG

Reverse: TTAGCCCTCCCACACATAACC

On the 5' end of the primers should be ~50 nts of sequence immediately upstream and downstream of the region to be modified, similar to the N-term tag.

Order an oligo to be used to 'rescue' the cassette insertion. Again, this should have 40-50nts homology on BOTH ENDS, surrounding the sequence that will be changed, and homologous to the sequences that will be surrounding the counter-selection cassette. This means your oligo will likely be 90-120nts long. Have it PAGE purified.

Also order 'checking' primers that anneal around where the cassette will insert.

Modification notes

Clones confirmed to have the cassette inserted should be tested for streptomycin sensitivity.

**Streak them onto plates containing streptomycin AS WELL AS another antibiotic for which a selectable marker is on the BAC. (otherwise the bacteria will simply lose the construct and grow)

**For this use an antibiotic encoding for the selectable marker closest to the modification site (i.e. if your construct has an EGFP tag linked to neo gene, and chloramphenicol resistance in the backbone, use kanamycin (neo)) This is VERY important to reduce selecting erroneous recombination events in the next step.

For the next step it is important to pick the clone that is the most sensitive on these plates. You will notice there is a variation, and most if not all clones will have some amount of revertants that have become strep resistant.

Use the best clone for the rescue step.

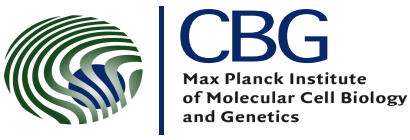
When transforming for the next Red E/T step (rescue), I usually use 5-10ul of a 50uM oligo stock per transformation. Also setup a transfection with induction, but no DNA.

Cells should be plated on plates containing streptomycin and an additional antibiotic (as mentioned above).

**You will usually only need to plate 5 ul or so from the entire transfection to get hundreds of colonies. The first time try different amounts.

You will also notice there will be HUNDREDS of colonies on the NO DNA plate as well. Sometimes there will be MORE colonies on the NO DNA plate even than the oligo transfected. It is still possible (and likely) to get correct integrants. Presumably the vast majority of the colonies on the NO DNA plate (and the oligo transfected) will be unwanted internal recombination events that popped out your cassette without the oligo rescue.

Because of this, the efficiency of getting a correct clone can vary from <1% to >50% depending on the gene or even the region within a gene being modified. Therefore it may be necessary to screen 100+ individual colonies (by colony PCR with 'checking' primers).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