

***Addition of C-terminal tag with selectable marker
(Quick and Easy BAC modification)***



Primer Design and PCR

Common C-terminal tagging cassettes currently in the lab include GFP, TAP, LAP, and FLAP (Flag-LAP). All have the neo resistance gene attached (resistant to kanamycin in *E. coli*)

Following are the sequences for each that should be at the 3' end of each primer:

EGFP

Forward: TCGAGCGGTCCCTCGGGTTCG

Reverse: TCAGAAGAACTCGTCAAGAAGG

TAP

Forward: GAAAAGAGAAGATGGAAAAAG

Reverse: TCAGAAGAACTCGTCAAGAAGG

LAP

Forward: GATTATGATATTCCAACACT

Reverse: TCAGAAGAACTCGTCAAGAAGG

FLAP

(same as LAP)

The 5' end of the primers should be 40-50 nts of homology immediately before (for the forward primer) and after (for the reverse primer) the stop codon

As an example, here is the sequence of mouse TPX2 around the stop codon (bold) and the corresponding primers for a 'LAP' tag:

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CATACTTCTCCTTCCCCTGAATTTGTAGTTGGGATCTATAAATCCTGTCCCCACCCTTCT
CCCCTAACAGACAGAACAACTGTGCACATAGATGTGTGCAGTCCACCTCAGGAACCCTGT
TTCTTATTCTACAGGTGCACAAGGCAAATCCAATACGGAAGTACGCAGCAGTGGAGGTGA
AATCTAGCGAGCTGCCTCTGACTGTGCCGGTGTCTCCTAAGTCTCCACTCGGTTCCAGTAG
AAGCTGCATCTATATCACAAGCATGGAGCACGCGTCTTCAGACTCACCCGCCATGTGT
GGAAACACACCTGATAACTAATGGATGCCGGGTGTTTTGAAGATGACTCTACACAGCAAC
TTTGAAGCTAATGAGCCTCCACTCATTTAGACTTTAACCAGACTTCTTGTAGTTATTTCC
TTCAGACAT
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Forward:

GCCTCTGACTGTGCCGGTGTCTCCTAAGTTCTCCACTCGGTTCCAGGATTATGATATTCCAACACTAC
T

Reverse:

GAGTCTGAAGACGCGTGCTCCATGCTTGTGATATAGATGCAGCTTTCAGAAGAACTCGTCAAGAA
GG

'Checking' primers should be ordered at the same time. These are simply short primers that anneal in the genomic DNA around the modification site, which will give different size products depending on if the modification has occurred or not.

*On BACs Don't forget to use the genomic sequence of the gene and not the mRNA/cDNA sequence when designing all primers

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For PCR I usually use the following reaction

- template (plasmid, BAC, PCR, etc.)
- 2.5 Primer 1 (10uM)
- 2.5 Primer 2 (10uM)
- dNTPs (10mM)
- 5.0 10x Buffer
- 0.5 poly (HiFi)
- 37.5 H₂O

and program (change as necessary)

- 94° 3min

then the following for 20 cycles:

- 94° 1min
- 60° 1min
- 72° 3min

then a final elongation:

- 72° 7min
- 4° 4ever

It's important to keep the cycles lower to avoid amplifying any introduced mutations too much. Similarly, a polymerase or mix with proofreading activity is better to avoid mutations (The MPI hifi mix works fine). I use 4 separate 50ul reactions and then PCR-purify them using a qiagen kit, and concentrate them by resuspending all together in a final volume of 50 ul.

From this I use 5-8 ul for one transformation.

Modification steps

I usually scale up a bit from the genebridges protocol. I usually use 4ml cultures for the induction/transformation. i.e. 4mls grown to OD₆₀₀ of ~0.3, then for induction add 125ul 10% L-arabinose, 1 hour. Spin down, resusp 1ml ice cold water, spin down in cold, resusp 1ml ice water, spin down and resusp in 100ul ice water. Use all of this for transformation (electroporation).

When plating after transformation, plate everything. Either all on one plate or have one with a 1:10 dilution. It is not uncommon to only get 3 transformants in the entire mix and for them all to be modified correctly. It is also not uncommon to get thousands.

Restreak 8-12 colonies from transformation. The following day do colony PCR with the checking primers to see if the tag was incorporated.

Now you may want to sequence your tag to make sure it did not pick up mutations through PCR before continuing. BACs are often troublesome to sequence off of. I have found the most reliable way to get decent sequencing off of a BAC is to make a miniprep of the BAC (as described in the Genebridges protocol), and then PCR off that the region to be sequenced. This PCR product can be used as sequencing template. Two separate PCR and sequence reactions for each sequence needed can be used to avoid finding mutations introduced in this last PCR step (they wouldn't be in both).

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