GENE BRIDGES
DNA ENGINEERING SPECIALISTS

Recombineering with Red/ET
Conditional Knock-out Constructs Service

Gene Bridges generates
Conditional Knock-out Constructs for Euro 9,990

Probably the easiest and most convenient method of generating targeting vectors is to let Gene Bridges do it for you. We apply Red/ET Recombination, a novel DNA engineering technology, to generate targeting vectors which fit your requirements precisely – down to the last nucleotide.

Conditional Knock-out constructs made by Red/ET Recombination include:

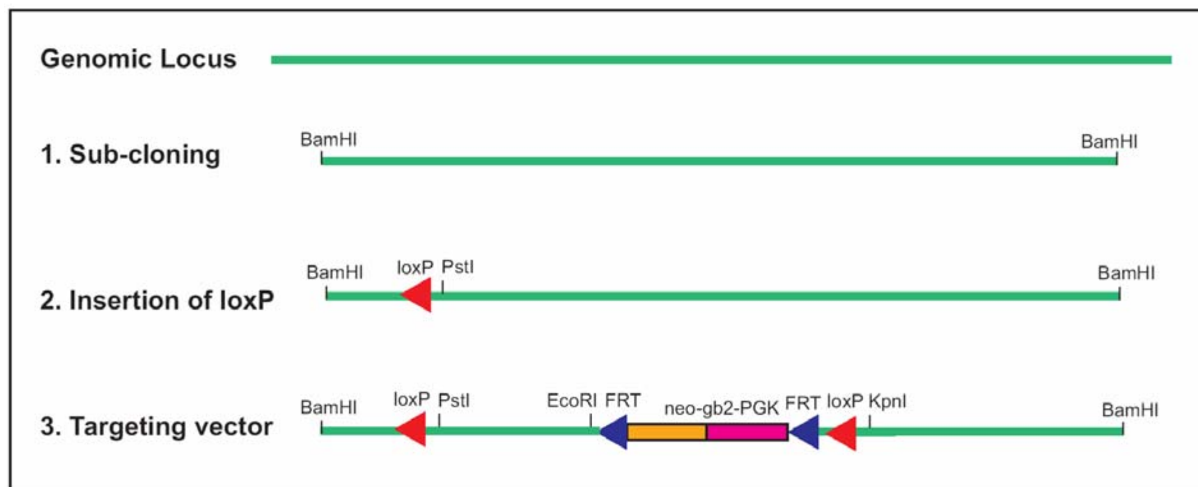
1. Sub-cloning of a fragment up to 18kb from a BAC into a “high copy” vector system provided by Gene Bridges

2. Insertion of a single loxP or FRT site into one location of the sub-cloned DNA

3. Insertion of a floxed or flrted PGK-gb2-neo cassette (kanamycin/neomycin resistance cassette under the control of a prokaryotic and a eukaryotic promoter) into a second location of the sub-cloned DNA

4. Verification check of all FRT, loxP sites and PGK promoter by sequencing

Exemplified Strategy - Targeting Vector Generation



Estimated timelines: 12 weeks from receipt of ordered BACs

You provide:

- Exact information about the gene to be targeted incl. accession number
- Length of desired homology arms to be inserted upstream and downstream of the functional elements to be inserted (e.g. 12kb upstream and 5kb downstream). Please indicate the maximum length of the locus to be sub-cloned (limited to 18kb).

You can choose:

- 1-2 additional restriction sites flanking the locus to be sub-cloned for later linearization of the targeting plasmid
- Exact insertion position of single loxP or FRT site (between nt “A” and nt “B”)
- Exact insertion position of a floxed or flrted PGK-gb2-neo cassette (between nt “C” and nt “D”)

In order to provide a targeting vector meeting your expectations, please provide Gene Bridges with the following information:

Gene Bridges will keep all information provided within this form strictly confidential!

Name ………………………………………… First Name ……………………………………………

Position….…………………………………………..…………………………………………..

Company/Institute…………………………………………………………………………………

Street………………………………………………………………………………………………

Zip/postal code.……………………………… City ……………………………………………………

State ………………………………………… Country ………………………………………………..

Tel.: ………………………………………….. Second Tel.: ………………………………...............

Fax: ………………………………………….. Email: ……………………………………….............

Project related data

1. Name of the gene to be modified ……………………………………………………………..
2. Accession number of the gene to be modified ……………………………………………………
3. Accession number of BAC (if it is available) ……………………………………………………
4. The locus the gene is mapped to (if it is available) ………………………………………………
5. Does the sequence or part of it belong to a multi-gene family:

   Yes **Don't know** **No**

6. Fragment to be sub-cloned. Please provide 100 nt sequence information of the ends of your insert (max. 18 kb).

   5'……………………………………………………………………………………………..

   3'……………………………………………………………………………………………..

   Additional restriction site(s) for subsequent separation of vector backbone.

   5'... 3'...

7. Position of single loxP or FRT site. Please check appropriate box.

   loxP **FRT**

   Please provide 100 nt sequence information flanking the site.

   5'………………………………………………………………………………………........

   3'……………………………………………………………………………………................

   Additional restriction site(s) (e.g. according to your southern blot strategy)

   5'..…. 3'...

8. Indication of cassette to be inserted and its orientation. As a standard we insert the PGK-gb2-neo cassette in opposite orientation of the open reading frame of the gene to be targeted. Please check appropriate box(es).

   FRT-PGK-gb2-Neo-FRT **FRT-PGK-gb2-Neo-FRT-loxP**

   loxP-PGK-gb2-Neo-loxP **same orientation as gene of interest**

   Please provide 100 nt sequence information flanking the site.

   5'………………………………………………………………………………………………………..

   3'………………………………………………………………………………………………………..

   Additional restriction site(s) (e.g. according to your southern blot strategy)

   5'.. 3'...

9. Your comments/additional explanations can be provided as additional text or diagram.

In case you experience problems completing this file or general questions regarding Gene Bridges services or products, please feel free to contact us:
contact@genebridges.com

Thank you for providing the necessary information.

Providing Gene Bridges with as much information/data as possible in advance will help us deliver your desired results quickly.

We will contact you shortly.

Experimental setup

We will order two independent BAC clones from the C57-BL/6J derived libraries RPCI-23 or RPCI-24 or from the 129Sv derived library bMQ covering the locus of interest. The BAC fragment will be sub-cloned from one of the two BACs into a "high copy" minimal vector containing a ColE1 origin of replication and an ampicillin resistance marker. This step is achieved by using Red/ET recombination. An additional restriction site flanking the insert for later linearization of the targeting vector can be included at the same time.

Insertion of a single loxP site:

1. Red/ET Recombination mediated insertion of a floxed cassette and
2. subsequent Cre-mediated deletion, leaving a single functional loxP-site. The loxP-site will be flanked by a 20 bp long T3 primer binding-site (plus an optional *Not*I site) at the 5'-end and a 20 bp long T7 primer binding-site (plus an optional *Xho*I site) at the 3'-end. The loxP site can be inserted together with additional restriction sites on either side e.g. for your southern strategy.

Insertion of a FRT-PGK-gb2-neo-FRT-loxP cassette by Red/ET Recombination:

The cassette will be flanked by a 20 bp long T3 primer binding site (plus an optional *Not*I site) at the 5'-end and a 20 bp long T7 primer binding-site (plus an optional *Xho*I site) at the 3'-end. The cassette can be inserted together with additional restriction sites on either side e.g. for your southern strategy.

FAQs

Q: Why is the overall size of the fragment which can be sub-cloned restricted to 18kb?

A: The maximum size to be sub-cloned into a plasmid depends on its inherent copy number. For the sub-cloning step we use a minimal vector containing a ColE1 origin of replication which gives rise to a copy number of more than 100 copies per cell. Due to this high copy number the maximum size of the insert which can be incorporated in the plasmid is 20 kb. The overall size of DNA to be sub-cloned during the targeting vector generation is restricted to a maximum of 18 kb because of the subsequent insertion of a 1.5 kb floxed neomycin cassette.

Q: Why do you order two different BACs for the generation of one vector?

A: Although the rate of chimerism and detected rearrangements in the BAC libraries mentioned above is relatively small (see Osoegawa K et al. 2000; Genome Research 10:116-128 and Adams DJ et al. 2005; Genomics 86:753-8), there is nevertheless a certain risk that one of the two BACs might be damaged, instable, chimeric or simply wrongly annotated. To prevent failure and subsequent delays, we perform the initial sub-cloning step with two different BACs in parallel.

Q: Can I choose BACs from a different source with a different genetic background?

A: We recommend selecting BACs from the currently the most reliable ones – as mentioned above. However, you may select BACs from another source. In this case we ask you to identify and order the BACs yourself and send them together with all necessary information including “how was the gene of interest mapped on this BAC”, overall insert size, vector backbone, mapping position, sequencing data etc.

Please submit the data via email to contact@genebridges.com.

You will be provided with a detailed cost and time estimate for your custom-designed project. The timelines and costs for individual project requests may differ from the offer above.

Q: Why are the *Not*I and *Xho*I sites flanking the cassettes listed as optional?

A: For internal controls the cassettes are all flanked by a *Not*I and an *Xho*I site. If you require either of these sites at other positions or for linearization or screening procedures, we can of course insert the cassettes without these restrictions sites at the ends.

Q: Why will the short DNA stretches of 20 bp flanking the single loxP site and the neo-cassette remain in the final targeting construct?

A: Our experience in cloning targeting vectors shows that including the sequences of the FRT and loxP sites in the oligos used for PCR amplification is possible but will result in extremely long oligonucleotides (up to 100bp and longer). The synthesis of very long oligonucletides is error-prone and expensive.
To keep the project costs low and the reliability high, the primers used to amplify the functional cassettes bind to the unique 20 bp long DNA stretch directly adjacent to the FRT or loxP site. In general these very short additional DNA stretches do not influence the downstream approach (for a detailed structure of the cassette see www.genebridges.com).

Remark

To perform Red/ET recombination homology arms 50bp in length must be added by PCR to the functional cassettes. The homology arms will be integrated in the oligonucleotides and added to the cassette by PCR reaction using a high-fidelity proofreading polymerase and a template from a sequence verified R6K-based plasmid. The primer binding site is located adjacent to the loxP or FRT site.
This results in long oligonucleotides: 50bp homology arms + 20bp binding primer sequence + approx. 10bp for additional restriction sites or other sequences. Of course these numbers may vary depending on the project.

Approximate experimental setup:

4x 80bp long oligos will be ordered to create the homology arms
4x 21bp long oligos will be ordered for PCR verification checks

How to order Conditional Knock-out Constructs for Euro 9 990?

Just complete this form and send it to contact@genebridges.com.

www.genebridges.com

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