Recombineering & Gap Repair Protocol

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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 (Warming *et al.*, 2005). The modified BAC can then be retrieved using gap repair and turned into a gene targeting vector. The BAC modifications are done using a modified bacterial strain, SW102. This strain is derived from DY380 (Lee *et al.*, 2001) 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 sole 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 single-stranded), 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.

The SW105 and SW106 strains (Warming *et al.*, 2005) are similar to SW102 except they carry the *Flp1* and the *cre* genes respectively under the control of the arabinose inducible promoter. The following protocols are also suitable for these strains. Induction of the *Flp1* and *cre* genes is described in Appendix D.

PROTOCOLS

1- Transferring BAC* into SW102.

1.1- BAC miniprep.

- Grow a 5 mL (10 mL) of BAC clone in LB/Chloramphenicol 12.5 µg.mL^{-1} overnight at 37°C.
- Harvest the bacteria by centrifugation in a 15 mL Falcon tube.
- Remove as much supernatant as possible.
- Resuspend the pellet in 250 µL (320 µL) P1 buffer with RNase A.
- Add 250 µL (320 µL) P2 buffer.

* BAC selection/ordering along with annotation display are described in **Appendix A**.

- Let stand at room temperature for 5 minutes
- Add 350 µL (450 µL) N3 buffer and mix by inversion.
- Let stand on ice for 5 minutes.
- Spin at 14000 rpm for 4 minutes.
- Transfer the supernatant in a clean tube.
- Spin at 14000 rpm for 4 minutes.
- Transfer supernatant in a clean tube.
- Add 750 µL Isopropanol.
- Let stand at room temperature for 10 minutes.
- Spin at 14000 rpm for 10 minutes.
- Wash with 1 mL 70% Ethanol.
- Air-dry (No speedvac).
- Add 25-50 µL of EB buffer and let dehydrate a few minutes.
- Use 1-2.5 µL for transformation. (ALWAYS USE FRESHLY)

1.2- Transformation of BAC from DH10B into SW102[†].

- Grow one colony overnight in LB without antibiotics at 30°C.
- Save 500 µL to make a glycerol stock (20% glycerol final concentration).
- Inoculate a culture at a 1/50 dilution

  15 mL of culture is required to obtain 40 µL competent cell which is necessary for each transformation. Calculate overnight culture and exponential culture volume in accordance.
- Grow up at 30°C for 3-4 hours until OD_{600} reaches 0.6.
- Put on ice/waterbath slurry for 5 minutes.
- Spin at 3200 rpm for 10 minutes at 0-4°C.
- Pour off all of the supernatant.
- Add 1 mL of autoclaved ice-cold MilliQ water
- Resuspend by gently swirling in ice/waterbath slurry. (This step takes a while)
- When resuspended, add 1 volume of ice-cold MilliQ water.
- Spin at 3200 rpm for 10 minutes at 0-4°C.
- Pour off all of the supernatant.
- Resuspend by gently swirling in ice/waterbath slurry.
- When resuspended, add 1 volume of ice-cold MilliQ water or 10% glycerol.

  Glycerol is useful if the cells are meant to be frozen.
- Spin at 3200 rpm for 10 minutes at 0-4°C.
- Pour off all of the supernatant.
- Add 1 mL of autoclaved ice-cold MilliQ water/10% glycerol.
- Transfer the cells in a 1.5-2 mL microcentrifuge tube.
- Spin at 4000 rpm for 10 minutes at 0-4°C.
- Pour off all the supernatant.
- Add ice-cold water or 10% glycerol so that the final volume is equal to 1/400 of original volume (25 µL/10 mL of original volume). Practically, add half of the final volume desired to obtain the correct final volume of resuspended bacteria (*e.g.* add 200 µL ddH2O/10% glycerol if final volume is 400 µL)
- Use 35-40 µL of electrocompetent cells for each electroporation in a 0.1 cm cuvette (Invitrogen) at 25 µF, 1.8 kV and 200Ω.
- After electroporation, the bacteria are recovered in 1 mL LB (Falcon) for 1 hour at 30°C in a shaking waterbath (*e.g.* Aquatron, INFORS).
- Transfer the cells into a 1.5 mL microcentrifuge tube.

[†] The protocol proposed uses electroporation to transfer the BAC DNA into SW102. Alternatively, chimiocompetent cells can be prepared (see Appendix D for protocol)

- Spin at 14000 rpm for 15 seconds.
- Discard most of the supernatant and resuspend in the left over.
- Plate on LB/Chloramphenicol 12.5 µg.mL^{-1} plate.
- Grow for 24-48 hours at 30°C.

1.3- Verification of BAC integrity.

This step is important to ensure that the BAC transferred into SW102 had not recombined.

- Extract BAC DNA from SW102 as described above.
- Digest 17.5 µL of BAC DNA from SW102 and DH10B with the appropriate enzyme (*e.g. Eco*RI or *Spe*I).
- PCR 1 µL of extracted DNA with primers corresponding to the homology arms.

2- Preparation of the recombination templates.

2.1- PCR amplification of homology arms using galK positive/negative selection.

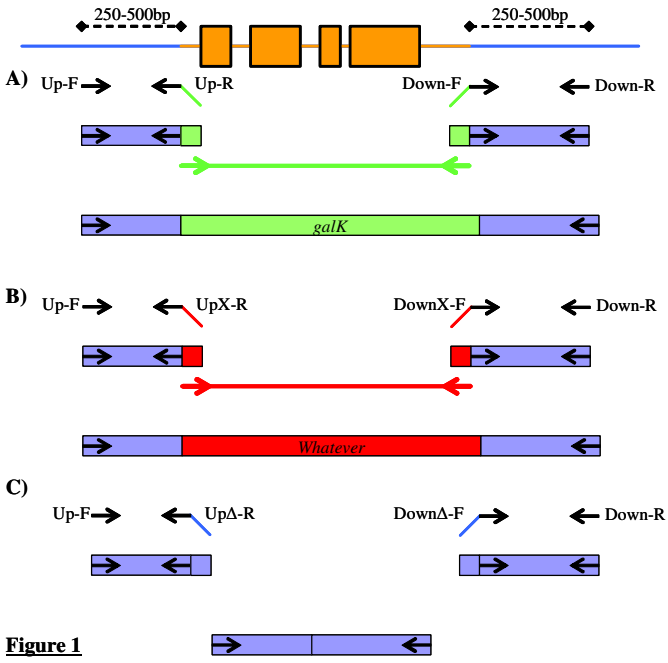


Figure 1

This method is used to either introduce a piece of DNA at a specific location into the BAC sequence or to delete a specific sequence without leaving any exogenous DNA sequence. It requires first the introduction a *galK* cassette (green) and its further removal. In this case, 2 sets of primers[‡] are needed per homology Figure 1. One set is used to generate the *galK* containing product flanked by 2 homology arms (Figure 1A; Up-F[§]/Up-R[§] and Down-F[**]/Down-R[§]); the second is used to generate a product that carries the desired modification (insertion of the "Whatever tag (red)" [Figure 1B; Up-F[§]/UpX-R and DownX-F/Down-R[§]] or clean deletion [Figure 1C; Up-F[§]/UpΔ-R and DownΔ-F/Down-R[§]]).

Experimental procedure

- PCR amplify 1 µL of BAC DNA with the different sets of primers using proofreading DNA polymerase (*e.g.* Expand High Fidelity PCR System, Roche Diagnostics).
- PCR amplify the Em7-*galK* cassette from pGALK[††] (REF) using proofreading DNA polymerase.
- After PCR reaction, add 1 µL of *Dpn*I to cleave BAC DNA into small fragments.

[‡] The Up-F and Down-R primers are always the same (See figure 1).
[§] Up-R**:** TGCCGATGATTAATTGTCAACAGG -- BAC sequence (Uppercase: *gal*K sequence)
[**] Down-F: AAGGAGCAGGACAGTGCTGA -- BAC sequence (Uppercase: *gal*K sequence)
[††] MTA is required to get this plasmid (http://www.recombineering.org)

- Run the PCR on a 1% agarose gel.
- Excise the band of interest with a scalpel blade.
- Gel-purify the PCR product (*e.g.* Qiagen QiaQuick Gel extraction kit).
- Dilute each of the PCR products to appropriate dilution. Use 10-50 ng of each PCR product as template.
- Generate your hybrid PCR product by mixing the appropriate purified PCR products with the leftmost and the right most primers used above (Up-F and Down-R).
- Run the reaction on a gel and gel purify the PCR product as above.
- Take OD. Concentration should be at least 100ng.µL^{-1}.

2.2- PCR amplification of homology arms using the galK/*3xP3*-miniwhite *cassette*

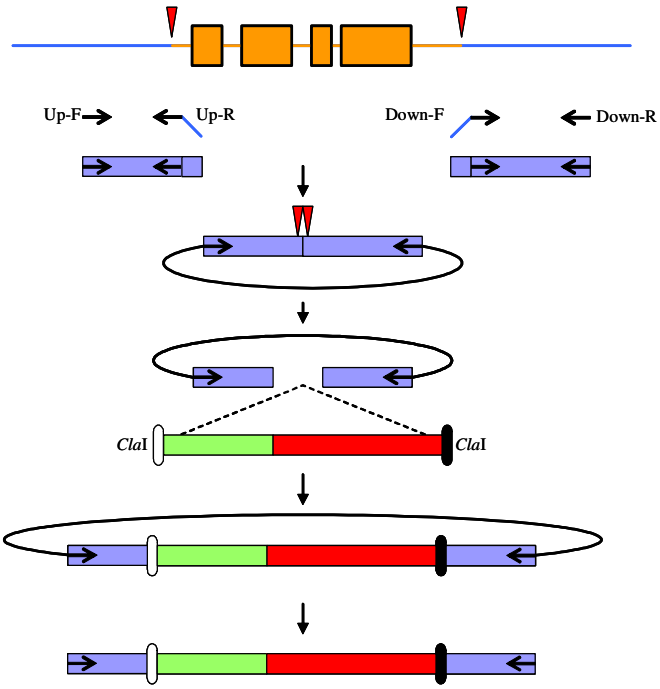


Figure 2

This method is used to introduce a fly dominant marker at a specific location within a BAC sequence taking benefit of the *galK* positive selection. In this case, the cassette to introduce contains the Em7-*galK* (green) and the 3xP3-*miniwhite* (red) genes flanked by two non-recombinogenic lox sequences suitable for Recombination mediated Cassette Exchange (loxP in white; lox2272 in black; \ (Oberstein *et al.*, 2005)). The disruption cassette contains a *Drosophila* gene which function cannot be assessed in bacteria (contrary to *gal*K). This cloning method, using a cloned and sequenced cassette, avoids the verification of the *miniwhite* gene that would be necessary if PCR amplified (see Figure 2).

The overall cassette is flanked by *Cla*I sites (see appendix B for restriction map). *Cla*I or compatible (*Acy*I and *Csp*45I) sites (red arrowheads) are present in the genome at a frequency of 1 every 600 bp. In case the case of RMCE, it is recommended to design homology arms containing such sites so that, after RMCE, the only modifications introduced would be the lox sites.

Experimental procedure

- PCR amplify the homology arms by PCR using 1 µL of BAC DNA with the different sets of primers using proofreading DNA polymerase (e.g. Expand High Fidelity PCR System, Roche Diagnostics).
- After PCR reaction, add 1 µL of *Dpn*I to cleave BAC DNA into small fragments.
- Run the PCR on a 1% agarose gel.

- Excise the band of interest with a scalpel blade.
- Gel purify the PCR product (*e.g.* Qiagen QiaQuick Gel extraction kit).
- Dilute each of the PCR products to appropriate dilution. Use 10-50 ng of each PCR product as template.
- Generate your hybrid PCR product by mixing the appropriate purified PCR products with the leftmost and the right most primers used above.
- Run the reaction on a gel and gel purify the PCR product as above.
- Digest the PCR product with appropriate enzyme and clone it into pBlueScript vector (or equivalent) or by T/A cloning.
- Digest the plasmid containing the homology arms by *Cla*I or compatible restriction site (see Design Strategy section for details).
- Clone into the *Cla*I digested plasmid the *Cla*I digested loxP-Em7-*galK*/3xP3-*miniwhite*-lox2272 cassette from the pKILL vector (see appendices for restriction map).
- If RMCE is considered, sequence the extremities of the clone to orientate the two lox sites.
- Excise the loxP-Em7-*galK*/3xP3-*miniwhite*-lox2272 cassette flanked by the two homology arms from the plasmid.
- Gel-extract the fragment of interest. Concentration should be at least 100ng.µL^{-1}.

2.3- Preparation of vector for BAC sequence retrieval

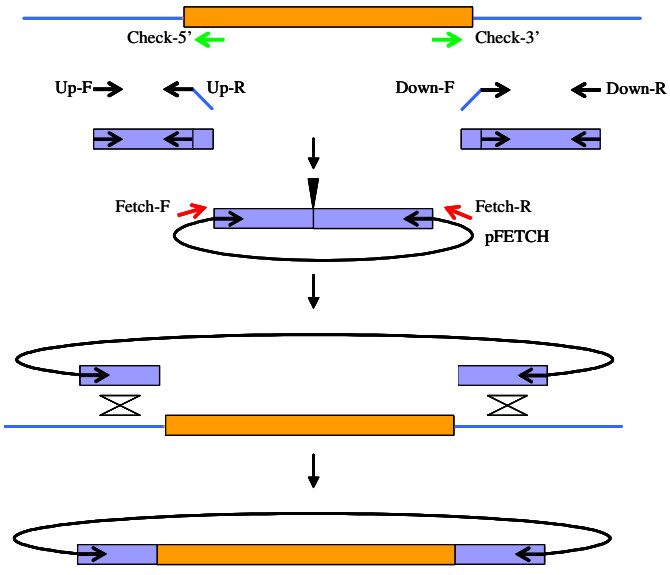


Figure 3

This method is used to subclone BAC sequence (orange) into appropriate vector that can be used for fly transgenesis. The vectors described here are of the pFETCH series and carry a ΦC31 attB site, a *Drosophila* dominant marker (3xP3-*eYFP* or 3xP3-*miniwhite*; see appendix B for restriction maps) and a multiple cloning site. The pFETCH#1 vectors are meant for transgenesis followed by homologous recombination using the ends-out Golic's method (Gong and Golic, 2003); the pFETCH#2 for RMCE (there is no dominant marker associated with the pFETCH#2 as the vector will be co-injected in embryos with a cre expressing helper plasmid); the pFETCH#3 vectors for transgenesis of large DNA constructs. It is possible to prepare these vectors with T-overhang for T/A cloning (see appendix D for protocol).

Experimental procedure

- PCR amplify the homology arms by PCR using 1 µL of BAC DNA with the different sets of primers using proofreading DNA polymerase (*e.g.* Expand High Fidelity PCR System, Roche Diagnostics).

- After PCR reaction, add 1 µL of *Dpn*I to cleave BAC DNA into small fragments.
- Run the PCR on a 1% agarose gel.
- Excise the band of interest with a scalpel blade.
- Gel-purify the PCR product (*e.g.* Qiagen QiaQuick Gel extraction kit).
- Dilute each of the PCR products to appropriate dilution. Use 10-50 ng of each PCR product as template.
- Generate your hybrid PCR product by mixing the appropriate purified PCR products with the leftmost and the right most primers used above.
- Run the reaction on a gel and gel purify the PCR product as above.
- Clone the hybrid PCR product using restriction enzymes or T/A cloning into the pFETCH vector
- Digest the plasmid using the enzyme which restriction site is located between the two homology arms for 1-2 hours at 37°C with Alkaline Phosphatase.

  The use of AP is meant to over-dephosphorylate the vector and thus minimize the vector self-ligation in E. coli.
- Gel purify the linearized vector. Concentration should be at least 100ng.µL^{-1}.

2.4- Induction of Recombineering function in SW102

- Grow an overnight culture of SW102 containing the BAC of interest at 30°C with chloramphenicol (12.5 µg.mL-1).
- Inoculate 30 mL of LB/chloramphenicol (12.5 µg.mL-1) at 1/50
- Grow the bacteria 3-4 hours at 30°C until OD600 ~ 0.6
- Transfer 15 mL in a 100 mL Erlenmeyer and incubate for 15 minutes exactly at 42°C in a shaking waterbath. Put the rest back at 32°C as uninduced control.

  At this point, it is very important that the heat shock is made in a shaking waterbath to ensure a constant temperature. If hot air is used, the temperature exchanges are less effective and the induction is not efficient.
- Prepare electrocompetent cells as describe above (**section 1.2**)
- Transform the induced and uninduced bacteria with ~ 300 ng of linear DNA (PCR product or linearized plasmid).

2.4.1- Addition of a galK-containing cassette.

- Let the bacteria recover in 1 mL LB at 30°C in a shaking waterbath for 1-1.5 hour
- Transfer the 1 mL of culture into an Eppendorf tube. Centrifuge at 13,200 rpm for 15 seconds at room temperature.
- Pipette off all the supernatant and resuspend the cells in 1 mL M9 salts.
- Centrifuge at 13,200 rpm for 15 seconds at room temperature.
- Pipette off all the supernatant and resuspend the cells in 1 mL M9 salts.
- Centrifuge at 13,200 rpm for 15 seconds at room temperature.
- After the second wash, the cells are resuspended in 1 mL 1X M9 salts.

  Washing the cells with M9 salts is necessary to remove any rich media from bacterial culture prior to selection on minimal medium in order not to interfere with selection and to reduce "hitch-hikers".
- Plate 100 µL of a serial dilution of induced bacteria (1/1, 1/10 and 1/100) on M63 minimal medium with galactose, leucine, biotin and chloramphenicol (see Appendix B). Uninduced bacteria display a high degree of cell death, resuspend them in 250/750 µL of M9 salts and plate 100 µL as control.
- Incubate the plates at 30°C for 2-4 days.

The colonies appearing should be Gal$^+$. In order to get rid of Gal$^-$ contaminant, streak a few clones on McConkey Agar containing galactose as sole source of carbon. Gal$^+$ clones will appear bright red/pink contrary to Gal$^-$ clones that will appear white/colorless. Usually, more than 90% of the clones tested are Gal$^+$.

At this point it is not required to check the BAC integrity as the only way to introduce the galK cassette is recombineering-mediated.

2.4.2- Removal of a galK-containing cassette.

- Let the bacteria recover in 1 mL LB at 30°C in a shaking waterbath for 4.5 hours.

  This long recovery is important to allow complete segregation of BACs by dilution. If a bacterium carries a galK+ and a galK- BAC, it will be counter selected.
- Transfer the 1 mL of culture into an Eppendorf tube. Centrifuge at 13,200 rpm for 15 seconds at room temperature.
- Pipet off all the supernatant and resuspend the cells in 1 mL M9 salts.
- Centrifuge at 13,200 rpm for 15 seconds at room temperature.
- Pipet off all the supernatant and resuspend the cells in 1 mL M9 salts.
- Centrifuge at 13,200 rpm for 15 seconds at room temperature.
- After the second wash, the cells are resuspended in 1 mL 1X M9 salts.

  Washing the cells with M9 salts is necessary to remove any rich media from bacterial culture prior to selection on minimal medium in order not to interfere with selection and to reduce "hitch-hikers".
- Plate 100 µL of a serial dilution of induced bacteria (1/1, 1/10 and 1/100) on M63 minimal medium with DOG, glycerol, leucine, biotin and chloramphenicol (see Appendix B). Uninduced bacteria display a high degree of cell death, resuspend them in 250/750 µL of M9 salts and plate 100 µL as control.
- Incubate the plates at 30°C for 2-4 days.

  Clones that grow from the uninduced control have lost the galK cassette by internal deletion of the BAC. This will give the background of BAC internal deletion in the induced sample.
- Analyze 12 DOG-resistant clones by *Spe*I digest and by PCR of the homology arms.

  It is important that the SpeI restriction pattern does not differ from that of the wild type BAC and that homology arms are still amplifiable by PCR. Clones which BAC displays obvious deletion will not be further analyzed.

2.4.3- BAC sequence retrieval .

- Let the bacteria recover in 1 mL LB at 30°C in a shaking waterbath for 1 hour.
- Centrifuge at 13,200 rpm for 15 seconds at room temperature.
- Spread all on LB containing the appropriate antibiotic for plasmid maintenance (usually Ampicillin 100 µg.mL-1)
- Incubate the plates at 30°C for 2-4 days.

  Clones that grow from the uninduced control are those in which the plasmid had self-ligate into the bacteria. This can be minimized by over-dephosphorylating the vector prior to transformation.
- Screen clones by PCR using Fetch-F/Check-5' and Fetch-R/Check-3' (see Figure 3).

  *Only correctly recombined plasmids should turn positive for **both** PCRs.*
- Extract BAC DNA for positive clones
- Perform *Spe*I or any other suitable enzyme to confirm sequence integrity. Compare fingerprint to modified BAC and *in silico* restriction pattern.
- Transform DH5α with obtained plasmids and plate on Ampicillin (100 µg.mL-1).

- Screen by PCR the bacteria containing only the correct recombined plasmid.
- Prep plasmid DNA for further use.

APPENDIX A: BAC ordering and annotation display

1- BAC ordering

1.1- Method #1

In the Flybase report for your gene of interest (http://flybase.net[‡‡]), click on the "**Gene region map**" hyperlink to visualize the genomic context. In the "Genome reagents" subclass of the "Tracks" panel located below the map, allow "**Tiling BAC**" to see which BAC(s) overlap the region.

Normally, most of the heterochromatin is covered by BAC. However, for some reasons (the sequencing status of the BAC or the BAC source), some BACs are not in the Flybase database and thus not displayed on genomic maps. For instance, no BAC or not all the BACs spanning the region are shown. In such case, try first to zoom out (around a 100kbp scale). If this doesn't work, try the second method.

1.2- Method #2

In the Flybase report for your gene of interest get the 3'- and 5'-UTR sequence of your GOI and BLAST it against the HTGS Drosophila database at NCBI. Go to the BLAST homepage (http://www/ncbi.nlm.nih.gov/blast) and click on the "**Fly**" hyperlink in the "Genomes" box. Then paste the UTR sequences (one at the time), choose the HTGS database in the Database list box and BLAST it.

Once you have selected your BAC of interest, you can order it (as stab stock) at Chori that handles the Drosophila BAC library (http://bacpac.chori.org/dromel98.htm). The cost is $25 per clone.

2- Annotation display

The Genepalette program (http://www.genepalette.org) is an open source program that is able to display genomic data from GenBank files. There are versions for all existing operating systems including MacOSX. This program is very convenient to visualize the genomic organization of a BAC and helps a lot designing primers for targeting or gap repair experiments. Once Genepalette is installed on your computer, there are 2 methods to import the information.

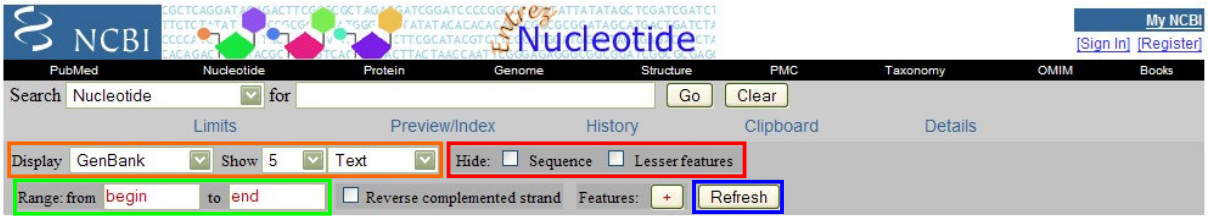
2.1- Method #1

Access the BAC sequence in ENTREZ nucleotide database. BAC sequences are not annotated in GenBank but the annotation can be gathered from the complete chromosome sequence. In order to do this, BLAST the BAC end against the chromosome sequence it originates from using bl2seq (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi). Use the BAC sequence as query and the chromosome GenBank Identifier (X: AE014298; 2L: AE014134; 2R: AE013599; 3L: AE014296; 3R: AE014297) as Subject. BAC extremities may correspond to repeated sequences. In such case, BLAST more internal sequences.

Once the BAC extremities have been mapped on the chromosome sequence, retrieve this sequence from the GenBank chromosome file (Figure 1). To do this: i) unclick "Hide" "sequence" and "lesser feature" in the upper box (red), ii) in the "range from … to" box, type the

[‡‡] All the hyperlinks are functional in this document and are shown as underlined blue text.

beginning and end of the sequence to retrieve (green), iii) click on refresh (blue) and iv) select "Text" in the Display box (orange). The file will appear as a text GenBank flat file which can be use as template in Genepalette.



In Genepalette, click on "**File**" menu, "**New sequence**", "**GenBank Flat File**". Paste the Flat File into the lower box and give a name to the sequence.

2.2- Method #2

As long as the working sequence is a small part of a BAC, sequence and annotation can be directly loaded from NCBI by Genepalette. In the "**Genome Tools**" menu, click on "**Load a sequence by GI#**". In the dialogue box, type the GenBank ID for the chromosome of interest. The program retrieves the genes that are present on the chromosome and prompt you to choose the genes to be displayed. Select the genes of interest by clicking in the "**include**" column and click OK. A dialogue box indicates how many genes will be loaded and the coordinates of the retrieved sequence relatively to the gene of interest. These coordinates may be changed manually before clicking OK.

The second method is easier than the first. However, in gap repair experiments, it may be useful to have the genomic organization of the complete BAC sequence rather than that of a fragment. In such case, the first method will be preferred. For other purposes (e.g. targeting) the second method is sufficient.

APPENDIX C: Media and reagents

M9 salts (1X) for 1L:

Na_2HPO_4	6 g	60g (10X)
KH_2PO_4	3 g	30g (10X)
NH_4Cl	1 g	10g (10X)
NaCl	0.5 g	5g (10X)

Autoclave at 121°C for 15 minutes.

M63 salts (5X) for 1L:

$(NH_4)_2SO_4$	10 g
KH_2PO_4	68 g
$FeSO_4 \cdot 7H_2O$	2.5 mg
Adjust to pH = 7 with KOH	

Autoclave at 121°C for 15 minutes.

Other:

Biotin	0.2 $mg.mL^{-1}$ (1:5000) [Filter sterile]
Galactose	20% in water (1:100) [Filter sterile]
Glycerol	20% in water (1:100) [Filter sterile]
2-deoxy-Galactose	20% in water (1:100) [Filter sterile]
Arabinose	20% in water [Filter sterile]
L-Leucine	10 $mg.mL^{-1}$ (1%) [Filter sterile]
MgSO4	1 M (1:1000) [Filter sterile]
Chloramphenicol	25 $mg.mL^{-1}$ (1:2000) [Filter sterile]
Ampicillin	100 $mg.mL^{-1}$ (1:1000) [Filter sterile]

M63-Agar plates for 300 mL/1 L:

- Autoclave 4.5 g/15 g agar in 240/800 mL H2O.
- Let Cool down a little.
- Add 60 mL/200 mL of autoclaved 5X M63 medium.
- Add 300 µL/1 mL $MgSO_4$.
- Adjust volume to 300 mL/1000 mL.
- Let cool down at 50°C (touchable)
- Add 3.3 mL/10 mL of carbon source (For DOG plate add Glycerol and DOG)
- Add 1.5 mL/5 mL Biotin
- Add 1.4 mL/4.5 mL L-Leucine
- Add 150 µL/500 µL Chloramphenicol.

MacConkey Indicator plate

Prepare MacConkey Agar (ref) according to the manufacturer's instruction. **It must be without carbohydrates.** Add galactose to 1% final concentration and Chloramphenicol 12.5 $\mu g.mL^{-1}$.

APPENDIX D: Alternative and accompanying protocols

1- Universal T/A cloning T-vector preparation

Purification of blunt-ended plasmid and addition of T-overhang

- In a 1.5 mL microcentrifuge tube, digest 10µg of plasmid with either
  a blunt-end restriction enzyme (e.g. EcoRV) [Preffered] or
  a 5' overhang-end restriction enzyme filled-in with Klenow polymerase
  Mix by gentle vortex and centrifuge briefly.
- Incubate at 37°C for 1-2 hours.
- Run 1-2 µL on gel to ensure complete digestion.
- Add 2 µL of 0.5 M EDTA to the tube at the end of digestion.
- Purify the vector using on Qiagen column (e.g. Quiaquick PCR Purification Kit)
- In a 1.5 mL microcentrifuge tube, add:
  - 46 µL of blunt-end digested plasmid
  - 15 µL of 5x TdT Buffer
  - 7.5 µL of CoCl2
  - 1.5 µL of 1 mM ddTTP§§
  - 5 µL of Terminal Transferase*** (25 U/µL)

  Mix gently and centrifuge briefly and incubate 1.5 hours at 37°C.

  The use of ddTTP prevents extension of a T-tail at the end of the DNA molecule. This could not be achieved with dTTP.

Purification and storage of T-vector

- Purify the T-tailed DNA solution either using column or by Phenol/Chloroform/Ethanol precipitation.
- In a 1.5 mL microcentrifuge tube, add:
  - 44µL of purified plasmid as above
  - 5 µL of 10X ligase buffer
  - 3 µL of T4 DNA Ligase††† (1 U weiss/µL)

  Mix well and centrifuge briefly. Incubate overnight at 16°C.

  This step is not absolutely required as T/A cloning will be efficient at 70% without any further purification. If the vector used does not allow blue/white screening after cloning, ligation/gel purification increases T/A cloning efficiency to 90%.
- Run a 1% agarose gel in TAE at 5V.cm-1
- Excise the band with a scalpel blade and gel purify it (QuiaQuick Gel Exctraction Kit)
- The vector molecules on which a single T or no T was added will either form concatemers or self-ligate. Their size will be different from that of linear T-vector that has a T at both extremities.
- Quantify purify T-vector and aliquot it at 25-30 ng.µL^{-1}. Usually, ligation are done with 50/60 ng of vector.

§§

*** Roche Diagnostics (ref: 03333566001)

††† Fermentas

2- DH10B/SW102 chimiocompetent cells

- Inoculate a culture from an ovenight culture at 1/50
- Grow until OD_{600} ~ 0.5-0.6
- Cool the culture in an ice/waterbath slurry
- Spin the bacteria at 4°C for 10 minutes at 3500 rpm
- Resuspend gently by swirling in an ice/waterbath slurry in 1/3.125 volume of CCMB80 buffer
- Incubate on ice for 20 minutes
- Spin the bacteria at 4°C for 10 minutes at 3500 rpm
- Resuspend in 1/12.5 volume of CCMB80 buffer.
- Incubate on ice for 20 minutes
- Aliquot the cells by 50 µL (volume requiered for transformation of standard vectors) or aliquot by 100 µL for BAC transformation.

CCMB80 Buffer:

KoAC	10 mM pH=7	(10 mL of a 1N stock solution)
$CaCl_2 \cdot 2H_2O$	80 mM	(11.8 gr/L)
$MnCl_2 \cdot 4H_2O$	20 mM	(4 gr/L)
$MgCl_2 \cdot 6H_2O$	10 mM	(2 gr/L)
Glycerol	10%	(100 mL/L)

Adjust pH DOWN to 6.4 with 0.1N HCl
Filter-sterilize and store at 4°C

Volumes:

Starting	1st wash	2nd wash
250 mL	80 mL	20 mL
100 mL	32 mL	8 mL

Colony PCR

- Inoculate each clone in 100 µL + appropriate antibiotic in a 96-well plate.
- Grow for 2/2.5 hours at appropriate temperature.
- Use 2.5 µL of each culture as template.
- Use 50 µL to inoculate culture of positive clones

REFERENCE

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Related papers and references: