

Recombineering Protocol for Constructing a Gene Targeting Vector for RMCE

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Kits and Reagents needed.

Kits:

1. BAC Library – for isolation of isogenic DNA for targeting construct
2. Qiagen Large Construct Kit – for expanding BAC clone
3. Qiagen PCR Purification Kit
4. Qiagen Gel Extraction Kit
5. Qiagen Mini-Prep Kit
6. Qiagen Maxi-Prep Kit
7. Qiagen Mega-Prep Kit

Bacterial cells:

1. EL350 – for recombineering transformation steps
2. DH5 α , XL1-blue, or other competent bacterial cell line – for non-recombineering transformation steps

Reagents:

1. LB media
2. Super Broth media
3. Chloroamphenicol
4. Kanamycin (working conc. = 12.5 ug/ml for BAC & 25 ug/ml for pDNA)
5. Ampicillin
6. Agar plates with appropriate antibiotic selection
7. Isopropanol
8. 70% ethanol

PCR reagents:

1. Amplitaq Gold
2. 10X Amplitaq Gold Buffer w/o Magnesium
3. 1.25 mM dNTP Premix

Oligonucleotides:

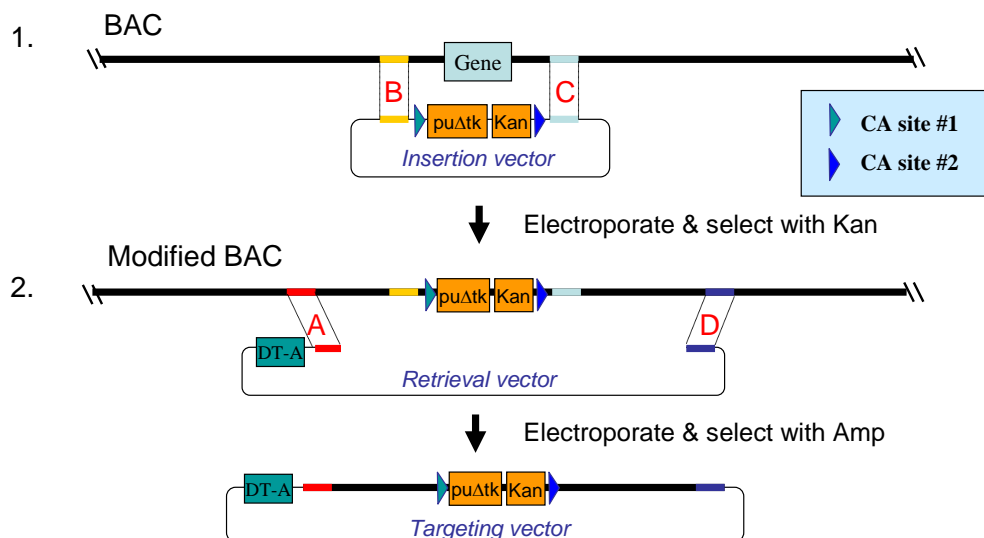
1. Eight pair to amplify the appropriate insertion and retrieval portions of the targeting vector (referred to in this protocol as fragments A, B, C, D)
2. Two pair to amplify gene sequence regions that lie upstream and downstream of the two targeting vector arms – to confirm isolated BAC clone and for Southern probe fragments
3. A minimum of two additional primers for sequence confirmation across the gene target locus

Preliminary design steps.

1. Determine gene of interest
2. Identify gene locus to be targeted

3. Select long and short targeting vector arms spanning a minimum of 8 and 4 kb respectively
4. Select the recombination strategy to be utilized for RMCE once gene targeting has been achieved
5. Design PCR primers to amplify ~500 bp homologous regions of the 5' and 3' ends of each targeting vector arm. These primers must introduce appropriate restriction enzyme (RE) sites on the 5' and 3'-ends of the matching gene specific arms to facilitate directional cloning into the appropriate insertion and retrieval vectors. Additionally, RE sites may be introduced in this design and amplification process that will enable Southern blot analysis of selected ES clones to screen for gene targeting events. Primer pairs should be designed as follows:
 - a. 1 oligo pair to amplify an ~500 bp fragment on the 5'-end of the left target arm (fragment A; primers geneA.F & geneA.R)
 - b. 1 oligo pair to amplify an ~500 bp fragment on the 3'-end of the left arm (fragment B; primers geneB.F & geneB.R)
 - c. 1 oligo pair to amplify an ~500 bp fragment on the 5'-end of the right arm (fragment C; primers geneC.F & geneC.R)
 - d. 1 oligo pair to amplify an ~500 bp fragment on the 3'-end of the right arm (fragment D; primers geneD.F & geneD.R)
6. Design PCR primer pairs to amplify probe fragments to screen for homologous recombination events at the target locus by Southern blot analysis. Probe fragments should be ~500 to 800 bp in length and lie upstream and downstream (external) of the targeting vector arms. These probes should be amplified from the isogenic DNA source used to construct the targeting vector. Oligo couples used to generate these probe fragments will also be used to select the appropriate BAC clone for constructing the targeting vector. Design oligo couples as follows:
 - a. 1 oligo pair to amplify a 5'-external probe fragment to be used for Southern Blot confirmation of targeted ES clones on the 5'-end
 - b. 1 oligo pair to amplify a 3'-external probe fragment to be used for Southern Blot confirmation of targeted ES clones on the 3'-end

Schematic illustrating two recombinering steps for targeting vector construction
 PCR amplified mini-gene fragments are noted (A, B, C, D)



Isolation of the Isogenic BAC DNA.

1. Generate probe
2. Hybridize BAC filter set
3. Order positive clones
4. Streak each BAC clone received on an agar plate treated with chloroamphenicol. Grow overnight at 37°C.
5. Pick a single well isolated colony for each BAC and grow overnight cultures in ~3 ml of LB + chloroamphenicol.
6. Prepare glycerol stock by combining 150 ul glycerol + 850 ul overnight BAC culture. Label and store at -80 °C
7. In a separate 0.5 ml Eppendorf tube, combine 40 ul of the overnight BAC culture + 160 ul of Nuclease-Free water. Heat 10 min. at 70 °C to lyse the bacteria. Microfuge 3 minutes at maximum speed; transfer supernatant to a clean tube. Lysed bacteria can be stored at -20 °C for later PCR requirements.
8. Perform PCR analysis to identify the appropriate BAC clone for targeting vector construction. Set up two separate PCR reactions for each BAC DNA using primer pairs designed to generate the 5' and 3' Southern probe fragments (see *Preliminary Steps, #6*).

Reactions are set up as follows:

33.5 ul Nuclease-Free water
5 ul 10X PCR buffer
8 ul 1.25 mM dNTPs
2.5 ul 20 uM 5' -primer
2.5 ul 20 uM 3' -primer
4 ul lysed BAC DNA
0.5 ul Amplitaq Gold
50 ul V_t

Cycling conditions for an ABI 9700 Thermocycler:

1 cycle at "94 °C x 4 minutes"
40 cycles at "94 °C x 1 minute, 60 °C x 30 seconds, 72 °C x 1 minute"
1 cycle at "72 °C x 7 minutes"
Hold at 4 °C until ready to remove.

PCR analysis:

Load 10 ul aliquots in a 1% mini-agarose gel (50 ml gel) along with an appropriate size marker and loading dye.

Identify BAC clones that yield amplicons of the appropriate size for both the 5' and 3' PCR screening reactions. These are the clones that span the entire region needed to construct the targeting vector and external screening probes.

Note: The appropriate 5' and 3' probe fragments amplified can be transformed into the TA cloning vector for expansion, retrieval, and purification to test on wild type ES cell DNA to determine if the expanded regions will serve as "good" Southern probe fragments.

BAC DNA expansion.

1. Grow a 5 ml starter culture of the desired BAC clone in LB + chloroamphenicol ~6 hrs. Use this starter to inoculate a 500 ml culture with the same media and antibiotic selection to grow overnight all at 37 °C.
2. On the following morning, perform BAC DNA extraction using the Qiagen Large Construct DNA Kit following the protocol exactly as outlined in the kit.
3. Resuspend isolated BAC DNA in a minimal volume of TE and allow to stand overnight in the final collection tube. Determine concentration by measuring A_{260} .

Constructing the Insertion and Retrieval Vectors.

A. Amplification of mini-gene fragments A, B, C, D

1. Using the isolated BAC DNA, PCR amplify the four gene specific flanking arm fragments (A, B, C, D) that will contain the appropriate RE cloning sites (see *Preliminary Steps, #5*).

Reactions:

5 ul 10X PCR buffer
8 ul 1.25 mM dNTPs
1.5 ul 20 uM primer.F
1.5 ul 20 uM primer.R
50 ng purified BAC DNA
0.5 ul Amplitaq Gold
Nuclease-Free water to 50 ul V_t

Cycling conditions for an ABI 9700 Thermocycler:

1 cycle at “94 °C x 6 minutes”
40 cycles at “94 °C x 1 minute, 60 °C x 30 seconds, 72 °C x 50 seconds”
1 cycle at “72 °C x 7 minutes”
Hold at 4 °C until ready to remove.

PCR analysis:

Load 10 ul aliquots in a 1% mini-agarose gel (50 ml gel) along with an appropriate size marker and loading dye.

2. Purify the mini-arm fragments (A, B, C, D) using Qiagen PCR Purification Kit following included protocol.
3. Digest the entire purified PCR sample with the appropriate REs to activate the introduced cloning sites. Run the digested samples in a 1% agarose gel and excise the digested fragments. Clean up using Qiagen Gel Extraction Kit. Purified mini-arm fragments can be stored at -20 °C until ready to sub-clone into the appropriate insertion or exchange vector.

B. Cloning mini-gene fragments

1. Select desired backbone vectors for Recombineering insertion and Recombineering retrieval into and out of the BAC source. Insertion backbone vector is typically a *pBluescript KS+* vector with two lox cassette acceptor sites flanking positive and negative selection cassettes for recombineering, ES

- homologous recombination, and RMCE events. The backbone retrieval vector is typically *pBluescript* with a negative selection marker for ES homologous recombination, i.e., *pBS.DT-A*.
2. Digest each backbone vector with the appropriate RE combinations to construct the basal insertion and retrieval plasmids (mini-gene fragments A & D will be cloned consecutively to create the basal recombineering retrieval vector while fragments B & C will be used to construct the basal LCA recombineering insertion vector to introduce the loxed locus into the BAC DNA).
 3. Follow standard DNA cloning protocols to introduce the purified PCR generated fragments into the desired cloning vectors. It is recommended all vectors be digested with an alkaline phosphatase enzyme to minimize negative vector background colonies resulting in more efficient miniprep screenings.
 4. Sequence across all cloned mini-gene fragments to confirm appropriate PCR amplification of the gene region of interest and to verify any LCA sites remain in tact.

Transfer BAC DNA into EL350 bacterial strain.

1. Grow EL350 bacterial cells in 5 ml of LB at 32°C overnight with shaking.
2. Collect the cells the next morning by centrifuging at 4 °C for 5 minutes; resuspend the pellet in 880 ul of ice cold water and transfer to an Eppendorf tube. Spin in microrcentrifuge at 5000 rpm for 4 minutes to pellet the cells.
3. Repeat wash 3 times with ice cold water.
4. Resuspend the cells in 50 ul ice cold water.
5. Mix the cells with ~100 ng of BAC DNA in an Eppendorf tube; transfer the cell-DNA mixture to a pre-cooled electroporation cuvette (0.1 cm gap) and perform electroporation using the pre-set program for bacterial cells.
6. Immediately add 1 ml of Super Broth (room temp.) to the cuvette, transfer to a 14 ml Falcon polypropylene round-bottom tube, and shake 1 hour at 32 °C.
7. Spread three chloroamphenicol resistant plates with 200, 100, and 50 ul of the bacterial cells, respectively. Incubate overnight at 32 °C. Note: It may be necessary to place these plates in a plastic container (such as a bag) to prevent over-drying if this is a potential problem with the incubator.
8. On the following day, pick several colonies and grow in 5 ml of LB with shaking at 32 °C overnight.
9. Mini-preps of BACs (reagents are from Qiagen DNA Kits). Collect the cells by centrifugation. Pour off media and resuspend bacterial pellet in 250 ul of P1 buffer; lyse by adding 250 ul of P2 buffer and incubate 5 minutes at room temperature; neutralize with 250 ul of P3 buffer – mix by inversion and microfuge on maximum speed for 5 minutes. Transfer supernatant to a new Eppendorf tube; precipitate the DNA by adding 0.7 ml (~480 ul) of isopropanol – mix by repeated inversion and microfuge 5 minutes. Pour off supernatant and wash once with 70% ethanol. Resuspend DNA in 50 ul of TE.
10. Mini-prep analysis. Digest mp-DNA and an aliquot of the original BAC with appropriate REs for comparison of digestion patterns to ensure transferred BAC is intact; separate digested fragments in a 0.9% agarose gel.

First recombinering reaction: Insertion of target region into BAC DNA

1. Digest about 2 ug of the LCA basal insertion vector (B.LCA-selection.C plasmid).
2. Gel purify the insert from the *pBluscript BS* backbone vector by running in a 1% agarose gel.
3. Use QIAquick Gel Extraction Kit to purify the DNA fragment; determine the concentration.
4. Grow EL350 cells containing the specific BAC in 5 ml of LB+Chloroamphenical overnight at 32 °C.
5. The next day transfer 1 ml of the overnight culture into a flask containing 20 ml of LB, incubate the mixture at 32 °C with shaking for 2 -2.5 hours
6. Transfer 10 ml of the culture to a new flask (store the remaining 10 ml on ice, these cells will be used as an negative control for recombinering); heat-shock the cells for 15 minutes at 42 °C to induce the production of the proteins required for recombinering; immediately chill the cells on ice.
7. Follow the protocol described in the previous section to prepare electro-competent cells.
8. Mix 50-100 ng of the linearized FragB.LCA-selection cassettes.FragC DNA with 50 ul of electro-competent cells and perform electroporation. Use EL350 cells without heat-induction as a negative control for recombinering.
9. Pour three Kanamycin-resistant plates with 200, 100, and 50 ul of the electroporated cells, respectively. Grow at 32 °C overnight.
10. On the following day, pick several kannmycin-resistant colonies and grow them overnight in 2 ml of LB+kanamycin with shaking
11. The next day, lyse 20 ul of each culture by mixing it with 180 ul of PCR-quality water and incubate at 70 °C for 10 minutes
12. Use 2-4 ul of the heat-inactivated bacterial culture in a 25-50 ul PCR reaction to verify the recombinering events for both the 5' and 3' junction regions.

Second recombinering reaction: Retrieval of DNA from modified BAC

1. Digest 0.5 ug of the basal retrieval vector (FragA.FragD vector) to linearize the plasmid. Note that excess amount vector DNA may lead to incomplete linearization of the vector, thus causing an unwanted high number of non-recombined colonies. Adding an alkaline phosphatase digestion step will greatly reduce the number of background clones obtained resulting in more efficient screening of mini-prep colonies.
2. Gel purify the insert from the plasmid backbone vector by running in a 1% agarose gel.
3. Use QIAquick Gel Extraction Kit to purify the DNA fragment; determine the concentration.
4. Resuspend the linearized DNA at 25-50 ng per ul with water or TE
5. Grow EL350 cells containing the modified BAC DNA generated from the first recombinering in 4 ml of LB+kanamycin overnight at 32 °C with shaking.
6. Prepare electro-competent cells following the protocol described previously
7. Electroporate 50 ng of the linearized retrieval vector into the prepared EL350 cells.

8. Pour three ampicillin-resistant plates with 200, 100, and 50 ul of the electroporated cells. Grow overnight at 32 °C.
9. Follow standard protocol to perform mini-preps, restriction enzyme digestions, and sequencing reactions to verify the retrieved DNA.

Stocking, expansion, and documentation of targeting vector.

1. Transform the confirmed mini-prep DNA into a non-temperature sensitive bacterial cell line for long term bacterial storage (-70 °C) and subsequent expansion.
2. Inoculate 5 ml of LB+antibiotic (ampicillin for DH5 α or XL1-blue competent bacteria) with re-transformed bacteria and grow for ~6 hours at 37 °C with shaking.
3. Transfer 5 ml culture to 500 ml of LB+antibiotic media and continue to grow overnight.
4. Using Qiagen Mega-Prep Kit, isolate purified targeting vector DNA from the 500 ml overnight culture.
5. Follow ES Core DNA Linearization Protocol to prepare DNA for electroporation into ES cells.
6. Targeting vector documentation: Construct a circular plasmid map noting all unique features of the targeting construct using VectorNTI or other appropriate software. Use this map and GenBank generated sequence to prepare a plasmid map document containing the (1) circular pDNA figure, (2) description of the targeting vector constructed, (3) name of person generating the construct and date completed (4) stocked bacterial and plasmid locations, (5) sequence of the plasmid in GenBank format.