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High-efficiency counterselection recombineering for site-directed mutagenesis in bacterial artificial chromosomes

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Supplementary File	Title
Supplementary Figure 1	Intramolecular recombination deletions in BACs after counterselection recombineering
Supplementary Figure 2	Sequences of *E. coli*, *Photorhabdus luminescens*, and 'optimized' *rpsL* gene.
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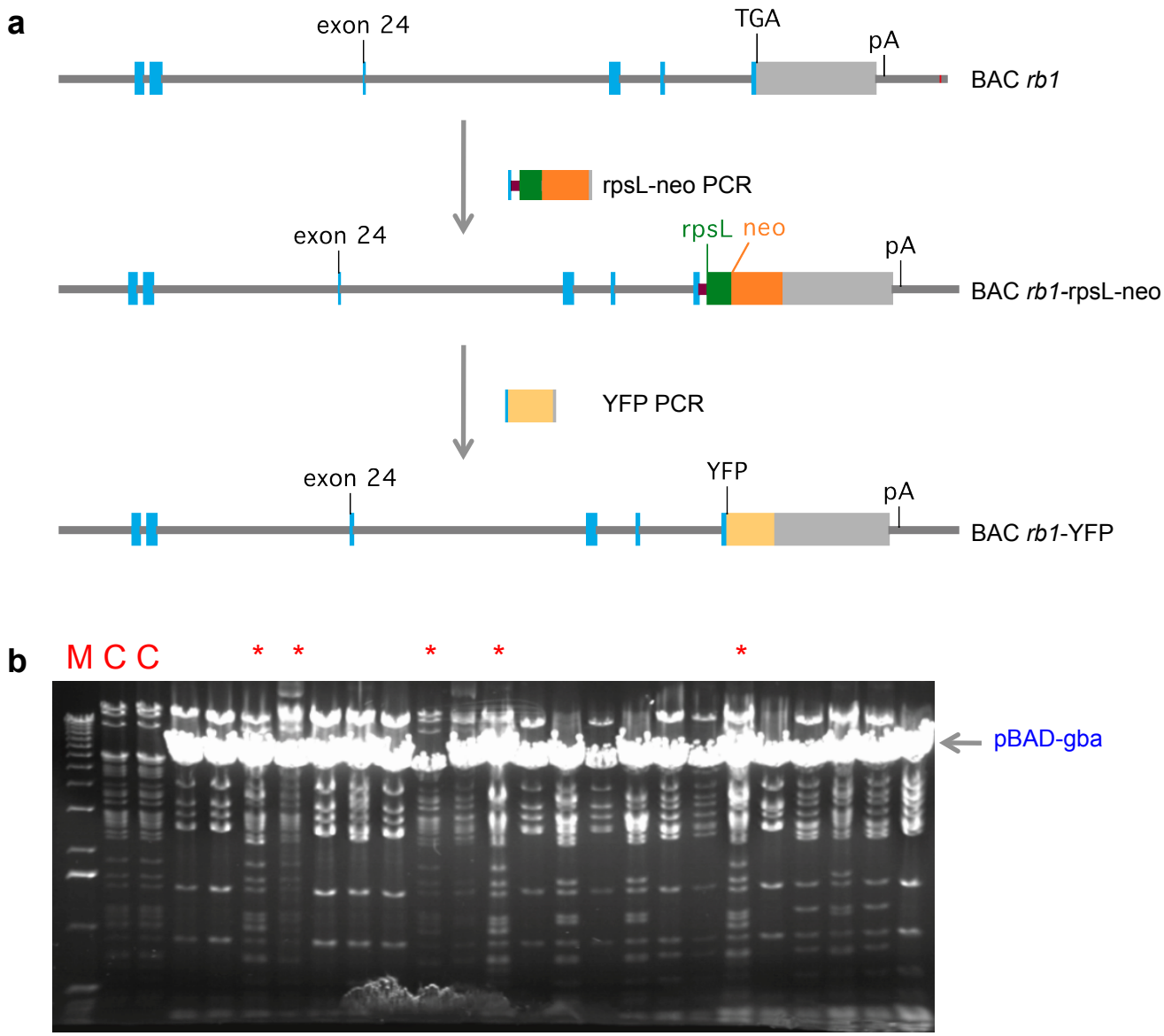
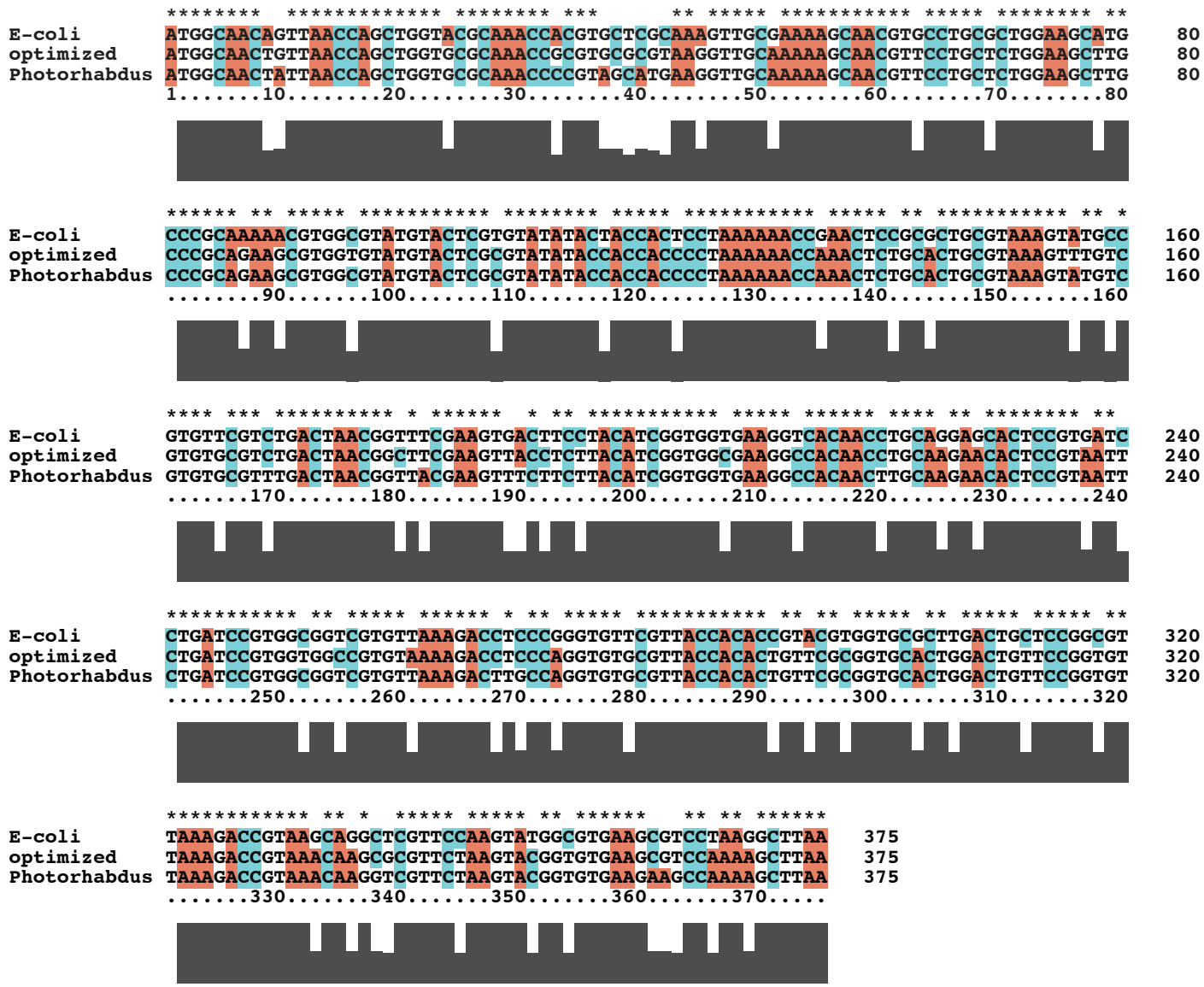
Supplementary Figure 1. Intramolecular recombination deletions in BACs after counterselection recombineering



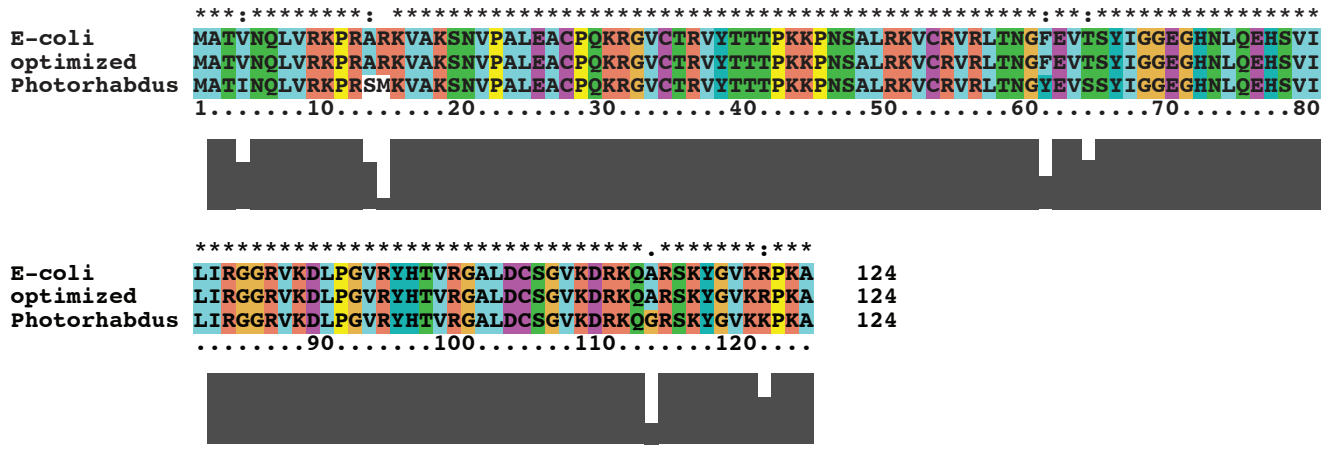
(**a**) Schematic illustrating insertion of a C-terminal YFP construct into a BAC through counterselection-based recombineering using Red gamma, beta, and alpha. (**b**) Restriction digest analysis of BAC minipreps of isolated candidates. pBAD-gba is the plasmid containing the recombineering proteins. M, molecular weight ladder. C, control lanes of unmodified BAC. Asterisks designate correct recombinants. 17 of 22 candidates contain unwanted deletions.

Supplementary Figure 2. Sequences of *E. coli, Photorhabdus luminescens*, and 'optimized' *rpsL* gene.

a



b



(**a**) Nucleotide sequence alignment of the *rpsL* gene from *E. coli* and *Photorhabdus luminescens*, with the optimized *Photorhabdus luminescens rpsL* sequence in the middle. There is no region of continuous identity longer than 13 bps. (**b**) Amino acid sequence alignment of the proteins of the above genes.

Supplementary Figure 3. Screenshots of the BACfinder2.0 online tool

a

BACfinder 2.0

Human gene TPX2 (ENSG00000088325) was found in the following BACs:

BAC name	BAC length	gene start	gene end	downstream
CTD-2343F13	98032	22996	85695	12337
CTD-2288O16	128948	23004	85703	43245
RP11-243J16	168859	33887	96586	72273
RP11-4O9	171640	52863	115562	56078
RP11-1149H15	173500	33886	96585	76915

BAC:
(By default will use the first one listed above)

Target transcript or protein Ensembl ID:

Sequence from target region:
(Target region must have at least 10 nt or 5 aa on each side of the desired mutation site and be from the transcript or protein entered above)

Mutated target region:
(must be same sequence as above with desired mutation)

Clear Submit

OR

BAC:
(By default will use the first one listed above)

Target transcript or protein Ensembl ID:

Target site position in transcript or protein:
(single position e.g. 173 or range e.g. 151-168)

Type of desired mutation:

- Deletion
- Insertion
- Replacement

Insertion/replacement sequence:

Clear Submit

b

BACfinder 2.0

BAC: CTD-2343F13

Cassette homology arm 1 (forward): TGGACTCACGCAGGCGCAGGAGACTACACTTCCCAGGAACTCCGGGCCGCG

Cassette homology arm 2 (reverse): GCCCTACAGACCGCAGCAATACCGGAAGTCAGAAGGAGGTACCAGCGAACA

Mutation oligo: GCCCTACAGACCGCAGCAATACCGGAAGTCAGAAGGAGGTACCAGCGAACATCGCGGCCC GGAGTTCCTGGGAAGTGTAGTCTCCTGCGCCTGCGTGAGTCCA

PCR primers around mutation:

5' Primer:

Sequence: TTCATAAGATCAGGGCAGGG
Tm: 60.029
Length: 20

3' Primer:

Sequence: GCCTAGTCGAATGCACCAAT
Tm: 60.103
Length: 20

Product length for primers pair: 491

(**a**) The first page allows for selection of BAC and input of desired mutation as either sequence or position, and as either nucleotides or amino acids. (**b**) The output page lists all oligonucleotides to o by counterselection.

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Supplementary Table 1. Oligonucleotide sequences used in this study

generation of TPX2 *rpsL-neo*
TGTTTTTCTGCACTAAGTTGGGGCAATGTCACAAGTCCCTACTACTggcctggtgatgatggcgggatcg
GTCTATATTTTCAGTATCCTCTTCAGCATCCAAAGATGAAAAATTGATAtcagaagaactcgtcaagaaggcg

generation of TPX2 *rpsL-bla*
TGTTTTTCTGCACTAAGTTGGGGCAATGTCACAAGTCCCTACTACTggcctggtgatgatggcgggatcg
GTCTATATTTTCAGTATCCTCTTCAGCATCCAAAGATGAAAAATTGATAttagccctcccacacataacc

TPX2 rescue (leading)
TCTGCACTAAGTTGGGGCAATGTCACAAGTCCCTACTACTGCCTCTGCAGCTGCCCCCACCGACTTTATCAATTTTTCATCTTTGGATGCTGAAGAGGATACTGAA

TPX2 rescue (lagging)
TTCAGTATCCTCTTCAGCATCCAAAGATGAAAAATTGATAAAGTCGGTGGGGGCAGCTGCAGAGGCAGTAGTAGGGACTTGTGACATTGCCCCAACTTAGTGCAGA

generation of TACC3 *rpsL-bla* (at siRNA resistance site)
CAGCAATGAAAAAAATACAGAAAATTGCGACTTCCTGTTTTCGCCACCAGAggcctggtgatgatggcgggatcg
TTGGCCAGGTTCTTGGGTGGCACATTTTCTTTCTGTGACACACGAAGAACttagccctcccacacataacc

TACC3 rescue (siRNA resistance)
AAAATACAGAAAATTGCGACTTCCTGTTTTCGCCACCAG AGGTAACGGGCCGTAGTAGCGTTCTTCGTGTGTCACAGAAAGAAAATGTGCCACCCAAGA

TACC3 rescue (siRNA resistance + S34A)
AAAATACAGAAAATTGCGACTTCCTGTTTTCGCCACCAGAGGTAACGGGCCGTAGTGCCGTTCTTCGTGTGTCACAGAAAGAAAATGTGCCACCCAAGA

generation of TACC3 *rpsL-bla* (at S552A S558A site)
GGCAGTCAGACCTGATCACTTGCCCTCTTGTCCCCAGTTTAAGGAGgggcctggtgatgatggcgggatcg
TCACCAGGACTGTCCCTCAGGAGGGGGTCGAACTTGAGGTATAAttagccctcccacacataacc

TACC3 rescue (S552A S558A)
GTCAGACCTGATCACTTGCCCTCTTGTCCCCAGTTTAAGGAGgCcGCCTTGAGGAAGCAagCCTTATACCTCAAGTTCGACCCCCTCCTGAGGGACAGTCCTGGT

generation of hAURKA *rpsL-bla* (at siRNA resistance site)
TCATTCGAAGAGAGTTATTCATAGAGACATTAAGCCAGAGAACTTACTTCTggcctggtgatgatggcgggatcg
ATACCTGGAAGATGGAGCATGTACTGACCACCCAAAATCTGCAATTTTAAGTTAGCCCTCCCACACATAACC

hAURKA rescue (siRNA resistance)
TCATTCGAAGAGAGTTATTCATAGAGACATTAAGCCAGAGAACTTACTTCTAGGTAGTGCAGGTGAATTAAAAATTGCAGATTTTGGGTGGTCAGTACATGCTCCATCTTCCAGGTAT

generation of hAURKB *rpsL-bla* (at siRNA resistance site)
ATGCCTTTGGCCTGAGGCCTCCTCTGTCTCTTCCCCCAGCCATCCCAACATggcctggtgatgatggcgggatcg
CCCGCGGGGGGCATACTCTAGAATCAAGTAGATCCTCCTCCGGTCATAAAATTAGCCCTCCCACACATAACC

hAURKAB rescue (siRNA resistance)
CCCGCGGGGGGCATACTCTAGAATCAAGTAGATCCTCCTCCGGTCATAAAAGTAATTATATAGTCTCAGAATGTTGGGATGGCTGGGGGAAGAGACAGAGGAGGCCTCAGGCCAAAGGCA

generation of hCKAP5 *rpsL-bla* (at siRNA resistance site)
TTAGGAAATGCCTCACTTAATCATTATTTTCCTTATCGACTAGGTTGGAGAggcctggtgatgatggcgggatcg
GCTAGCTGGGTAGACAAGGCACATCCGGTTCAGGATGGCACGAACATCTTTTTAGCCCTCCCACACATAACC

hCKAP5 rescue (siRNA resistance)
TTAGGAAATGCCTCACTTAATCATTATTTTCCTTATCGACTAGGTTGGAGAGCCTAAAGACGTGATAAGAAAAGATGTTCGTGCCATCCTGAACCGGATGTGCCTTGTCTACCCAGCTAG

generation of hMAD2L1 *rpsL-bla* (at siRNA resistance site)
TGAAAAATGGGAAGAGTCGGGACCACAGTTTATTACCAATTCTGAGGAAGTggcctggtgatgatggcgggatcg
ATTGACAGGAATTTTGTAGGCCACCATGCTATTTACTTTGTGGATTGTAGTTTAGCCCTCCCACACATAACC

hMAD2L1 rescue (siRNA resistance)
TGAAAAATGGGAAGAGTCGGGACCACAGTTTATTACCAATTCTGAGGAAGTGAGGTTAAGGAGTTTCACAACTACAATCCACAAAGTAAATAGCATGGTGGCCTACAAAATTCCTGTCAA

generation of mAURKA *rpsL-bla* (at siRNA resistance site)
CGGCGTTCTCTGCTATGAGTTCCTAGTGGGGATGCCTCCTTTCGAGGCACAggcctggtgatgatggcgggatcg
CCAGCCCTCGGCAGAAGCCTGACAGCAGCAGCTCACCCGAGATATCCTTCTTTAGCCCTCCCACACATAACC

mAURKA rescue (siRNA resistance)
CCAGCCCTCGGCAGAAGCCTGACAGCAGCAGCTCACCCGAGATATCCTTCTATATGTTTCTTGATAGGTATGTGCCTCGAAAGGAGGCATCCCCACTAGGAACTCATAGCAGAGAACGCC

generation of mCDC20 *rpsL-bla* (at siRNA resistance site)
ACCTTACATCTGTGACCCTGTGGTCATTCCTCTGCAGCTCTGGGATGTGCAggcctggtgatgatggcgggatcg
ATAGCTGTTCCAACTGAGGGAGCTTACTCGAGCGGAGTGACTGGTCATGTTTTAGCCCTCCCACACATAACC

mCDC20 rescue (siRNA resistance)
ACCTTACATCTGTGACCCTGTGGTCATTCCTCTGCAGCTCTGGGATGTGCAACAACAAAAGAGGTTAAGGAACATGACCAGTCACTCCGCTCGAGTAAGCTCCCTCAGTTGGAACAGCTA

generation of hGTSE1 *rpsL-bla* (at siRNA resistance site)
GGCAGCCCAAGCTGCCAAGCCTGAAGACCCTCGGAGCCAGGGCGTGGAAAG**ggcctggtgatgatggcgggatcg**
GGGGCTTTTCTTCATTTCCTTTTCTTTCTCAAAGAGGTTTATTTTTAATTTTTAGCCCTCCCACACATAACC

hGTSE1 rescue (siRNA resistance)
GGCAGCCCAAGCTGCCAAGCCTGAAGACCCTCGGAGCCAGGGCGTGGAAAGGTTTATCCAAGAAAGTAAATTAAAAATAAACCTCTTTGAGAAAGAAAAGGAAATGAAGAAAAGCCCC

generation of hTPX2 *rpsL-bla* (at siRNA resistance site)
ttggagaataagttactggggaagaatggaactggagggctttttca**ggcctggtgatgatggcgggatcg**
cttagctggtttcaaaggtgtgacaatagcttgctgaagattagccttTTAGCCCTCCCACACATAACC

hTPX2 rescue (siRNA resistance)
Ataagttactggggaagaatggaactggagggctttttca**a**ggtaa**g**ac**a**cc**a**ct**c**cg**c**aaggctaatcttcagcaagctattgtcacacctttgaaac

Supplementary Protocol 1. BAC mutagenesis/counterselection

BAC selection, ordering, and storage

BACfinder (http://www.mitocheck.org/cgi-bin/BACfinder) and BACfinder2.0 (http://www.mitocheck.org/cgi-bin/BACfinder2) will automatically provide a list of BACs containing a given gene of interest (mouse or human), and highlight those on which more than 10kb of genomic DNA are retained on both sides of the gene of interest.

Individual BACs may be obtained from a number of sources, including Children's Hospital Oakland Research Institute (CHORI) (http://bacpac.chori.org). They are delivered in a bacterial host and should be stored as bacteria at -80C.

For detailed protocols on recombineering-mediated introduction of protein tags onto genes of interest see Ciotta et al., Hofemeister et al, Poser et al.

Oligonucleotide design

BACfinder2.0 (http://www.mitocheck.org/cgi-bin/BACfinder2) is a web-based tool designed to provide sequences of oligonucleotides required for counterselection-based recombineering.

Upon visiting the BACfinder2.0 website, enter the gene of interest in the search box. Although the search box accepts various gene names, it is best to use an Ensembl gene ID (ENSG) consistent with the Ensembl genome assembly version used by

BACfinder2.0 as reference (indicated on the first page), to avoid mistakes caused by ambiguities in gene names.

At the top of the following page BACs containing the selected gene are displayed. A link to the selected gene in the reference Ensembl web site is provided and each BAC name is a link to a view of the BAC in the UCSC genome browser.

The rest of the page offers two options for inputting the desired mutation in the gene of interest: either by sequence or by positional information. For either option, start by selecting a BAC from the list. If no BAC name is entered, the first BAC from the list is automatically selected. Next, enter the Ensembl ID of the targeted transcript or protein. Again, this ID has to be consistent with the Ensembl version used as a reference.

In the first option, the targeted sequence and its mutated version can be entered. For this option, the targeted sequence must be taken from the selected transcript or protein and contain enough flanking material (at least 10 nucleotides or 5 amino-acids) on each side of the mutation site.

In the second option, the target site is identified by its position in the selected transcript or protein and a type of mutation is selected. Sequences to be inserted or used as replacement can be entered in the next box.

After a modification option is entered, the following page lists required oligonucleotide sequences. First, the sequences “Cassette homology arm 1 (forward)” and “Cassette homology arm 2 (reverse)” are given. These sequences will go on the 5’ end of the oligonucleotide used to PCR amplify a given counterselection cassette. The

3’ ends will contain a sequence specific to the cassette used. For cassettes used in this study, the corresponding sequences to add are:

rpsL-neo cassette
ggcctggtgatgatggcgggatcg for ‘Cassette homology arm 1 (forward)’ and
tcagaagaactcgtcaagaaggcg for ‘Cassette homology arm 2 (reverse)’

rpsL-bla cassette
ggcctggtgatgatggcgggatcg for ‘Cassette homology arm 1 (forward)’ and
ttagccctcccacacataacc for ‘Cassette homology arm 2 (reverse)’

Order these oligonucleotides with HPLC purification.

In the next box the “mutation oligo” sequence is given. This is the oligonucleotide to be transformed in the second recombineering step. When ordering, it is important to have phosphothioate bonds made in the first two positions (5’ end). Because of the length of this oligonucleotide it is also important to have it PAGE-purified.

Finally, sequences are given for primers that will be used to check for integration of the cassette of interest and the following replacement by the mutation oligo by colony PCR.

It is always a good idea to double check that the various oligonucleotides returned by BACfinder2.0 are consistent with the desired effect.

Chemicals

L-arabinose stock solution: 10% L-arabinose in ddH20; freeze aliquots at -20ºC
L-rhamnose stock solution: 10% L-rhamnose in ddH20; freeze aliquots at -20ºC

Antibiotics

Chloramphenicol (Cm) Stock solution: 30mg/ml in ethanol
Tetracycline (Tet) Stock solution: 10mg/ml in 75% ethanol
Kanamycin (Km) Stock solution: 40mg/ml in ddH2O
Streptomycin (Strep) Stock solution: 50mg/ml in ddH2O

Liquid Media

LB medium
LB+Cm: LB medium + 15ug/ml Chloramphenicol
LB+Cm+Tet: LB medium + 15ug/ml Chloramphenicol + 3ug/ml Tetracycline

Plates

LB+Cm: LB medium + 15ug/ml Chloramphenicol
LB+Cm+Tet: LB medium + 15ug/ml Chloramphenicol + 3ug/ml Tetracycline
LB+Cm+Tet+Km: LB medium + 15ug/ml Chloramphenicol + 3ug/ml Tetracycline + 15ug/ml Kanamycin
LB+Cm+Strep: LB medium + 15ug/ml Chloramphenicol + 100ug/ml Streptomycin

PCR reagents

High-fidelity polymerase
dNTPs

Equipment

PCR machine
Benchtop microcentrifuge
Microcentrifuge tube shaker

Before Starting

Most bacterial strains containing BACs are streptomycin resistant. Test for resistance before starting by streaking them on LB+Cm+Strep plates.

Make a DNA prep of pABRG using a standard plasmid purification protocol/kit.

Transform pABRG into bacteria harboring BAC of interest.

Day 1

Start an overnight culture of bacteria containing the BAC of interest in 1.0 ml LB+Cm in a microfuge tube. Puncture a hole in the lid for air. Incubate at 37°C overnight with shaking.

Day 2

Before starting: Chill ddH2O, electroporation cuvettes and benchtop centrifuge. (Or keep all at 4ºC)

1. Inoculate 1.4ml LB+Cm in a microfuge tube with 30ul of the fresh overnight culture and grow for 2-3 h at 37ºC, shaking at 1000 rpm.

2. Prepare the cells for electroporation: Centrifuge for 30 sec at 11,000 rpm in a cooled microfuge benchtop centrifuge (4ºC). Discard the supernatant by quickly tipping out the supernatant twice, and place the pellet on ice. Resuspend the pellet with 1 ml chilled ddH2O, pipetting up and down three times to mix the suspension. Repeat the centrifugation and resuspend the cells again. Centrifuge and tip out the supernatant once more; 20 to 30 µl will be left in the tube with the pellet. Resuspend cells and keep the tube on ice.

3. Add 1 ul (about 10–100 ng) of pABRG to the cell slurry in the microfuge tube. Mix briefly and keep on ice. Transfer the cell suspension from the tube to the chilled electroporation cuvette.

4. Electroporate at 1350 V, 10 F, 600 Ohms. This setting applies to an Eppendorf Electroporator 2510 using a 1 mm electroporation cuvette. Other devices can be used, but 1350 V and a 5 ms pulse are recommended.

6. Resuspend the electroporated cells in 1 ml LB medium without antibiotics and return them to the microfuge tube.

7. Incubate at 30ºC for 70 min, shaking at 1000 rpm. (pABRG will be lost at 37ºC).

8. Using a small loop, plate 30 µl cells on LB+Cm+Tet plates. Incubate the plates at 30ºC overnight.

Integrate counterselection cassette into site of interest

Day 3

Generate the counterselection cassette with homology ends by PCR by setting up three tubes with the following reaction:

0.4µl template DNA (0.1-1.0 mg/ml)
2.5µl "Cassette Homology Arm Forward" primer (10uM)
2.5µl "Cassette Homology Arm Reverse" primer (10uM)
1.0µl dNTPs (10mM)
5.0µl 10x polymerase PCR Buffer
0.5µl polymerase (high fidelity)
38.1µl H2O

50.0µl (total volume)

Use the following program to start:

94º 3min
followed by 8 cycles of:
94º 1min
58º 1min
72º 2min 30sec

followed by 15 cycles of:

94º 1min

64º 1min

72º 2min 30sec

and a final elongation:

72º 7min

4º (hold)

(conditions should be adjusted as necessary due to differences in primer sequences, etc.)

After checking for product on an agarose gel, combine the three reactions/products, purify using a standard PCR-purification kit, and concentrate by resuspending all in 50µl ddH20 final.

Set up overnight cultures by picking colonies from the pSC101-BBRG transformation plate and inoculate one microfuge tube containing 1.0 ml LB+Cm+Tet. Puncture a hole in the lid of the tubes for air. Incubate the cultures while shaking at 30ºC overnight.

Day 4

Before starting: Chill ddH2O, electroporation cuvettes and benchtop centrifuge. (Or keep all at 4ºC)

1. Set up two microfuge tubes containing fresh 1.4 ml LB+Cm+Tet and inoculate with 30µl of fresh overnight culture. (one will serve as a "no DNA" control).

2. Culture for about 2 h at 30ºC, shaking at 1100 rpm until OD600 = ~ 0.3.

3. Induce expression of Red gamma and Red beta: Add 50µl of 10% L-arabinose and 50µl of 10% L-rhamnose (final concentration each of 0.3–0.4%). Incubate at 37ºC with shaking for 50 minutes.

4. Prepare the cells for electroporation: Centrifuge for 30 sec at 11,000 rpm in a cooled microfuge benchtop centrifuge (4ºC). Discard the supernatant by quickly tipping out the supernatant twice, and place the pellet on ice. Resuspend the pellet with 1 ml chilled ddH2O, pipetting up and down three times to mix the suspension. Repeat the centrifugation and resuspend the cells again. Centrifuge and tip out the supernatant once more; around 30 µl will be left in the tube with the pellet. Resuspend cells and keep the tube on ice.

5. Add 2-3 µl of the counterselection cassette purified PCR product to the cell slurry to one of the microfuge tubes and pipette the mixture into the chilled electroporation cuvettes.

6. Electroporate at 1350 V, 10 F, 600 Ohms. This setting applies to an Eppendorf Electroporator 2510 using an electroporation cuvette with a slit of 1 mm. Other devices can be used, but 1350 V and a 5 ms pulse are recommended.

7. Add 1 ml LB medium without antibiotics to the cuvette. Mix the cells carefully by pipetting up and down and pipette back into the microfuge tube. Incubate the cultures at 37ºC with shaking for 70 minutes. Recombination will now occur.

8. Streak the cultures with a loop onto LB+Cm+Tet+Km plates (for an rpsL-neo cassette; for rpsL-bla use Amp instead of Km). Incubate the plates at **30ºC** overnight

to keep pSC101-BBRG in the host strain (it wil be lost at 37C). Incubate 24-36 hours or until colonies are large enough to pick. There should not be any colonies on the 'no DNA' plate.

Day 6

9. Restreak 8-10 colonies to a new LB+Cm+Tet+Km plate and incubate overnight at 30ºC.

Day 7

10. Restreak candidate integrants to LB+Cm+Strep plates to test for sensitivity to streptomycin and incubate at 37 °C overnight.

11. Perform colony PCR on candidate integrants (and the original BAC as a control) to check for presence of counterselection cassette. Add a small amount of each bacteria to the following reaction:

1.5µl "checking-up" primer (10uM)
1.5µl "checking-down" primer (10uM)
0.6µl dNTPs (10mM)
3.0µl 10x polymerase PCR Buffer
0.3µl polymerase (high fidelity)
23.1µl H2O

30.0µl (total volume)

Use the following program to start:

94º 3min
followed by 25 cycles of:
94º 1min
59º 1min
72º 3min
and a final elongation:
72º 7min
4º (hold)

(conditions should be adjusted as necessary due to differences in primer sequences, etc.)

Correct integrants should have a PCR product that is approximately 1.3kb larger than the product resulting from an unmodified BAC (without other products).

Setup overnight cultures of all colony PCR positive integrants for the next step

Day 8

Before starting: Chill ddH2O, electroporation cuvettes and benchtop centrifuge. (Or keep all at 4ºC)

Check candidate bacteria on the LB+Cm+Strep plate. Compare to the PCR result. For the next step take the integration candidate that is correct by PCR analysis and is the most sensitive to streptomycin (a few single cells may give rise to small colonies due to spontaneous mutations).

Recombine oligonucleotide with mutation of interest to replace counterselection cassette

1. Set up a microfuge tube containing fresh 1.4 ml LB+Cm+Km+Tet and inoculate with 30µl of fresh overnight culture of bacteria with correct cassette insertion in the BAC.

2. Culture for about 2 h at 30ºC, shaking at 1100 rpm until OD600 = ~ 0.3.

3. Induce expression of Red beta (only): Add 50µl of 10% L-arabinose (final concentration of 0.3–0.4%). Incubate at 37ºC with shaking for 50 minutes.

4. Prepare the cells for electroporation: Centrifuge for 30 sec at 11,000 rpm in a cooled microfuge benchtop centrifuge (4ºC). Discard the supernatant by quickly tipping out the supernatant twice, and place the pellet on ice. Resuspend the pellet with 1 ml chilled ddH2O, pipetting up and down three times to mix the suspension. Repeat the centrifugation and resuspend the cells again. Centrifuge and tip out the supernatant once more; 20 to 30 µl will be left in the tube with the pellet. Resuspend cells and keep the tube on ice.

5. Add 1 µl of the "mutation oligo" (at 50µM concentration) to the cell slurry to one of the microfuge tubes and pipette the mixture into the chilled electroporation cuvettes.

6. Electroporate at 1350 V, 10 F, 600 Ohms. This setting applies to an Eppendorf Electroporator 2510 using an electroporation cuvette with a slit of 1 mm. Other devices can be used, but 1350 V and a 5 ms pulse are recommended.

7. Add 1 ml LB medium without antibiotics to the cuvette. Mix the cells carefully by pipetting up and down and pipette back into the microfuge tube. Incubate the cultures at 37ºC with shaking for 70 minutes. Recombination will now occur.

8. Streak ~100µl from the cultures with a loop onto LB+Cm+Strep plates. Incubate the plates at 37ºC (pABRG plasmid will now be lost prior to BAC DNA preparation). Incubate overnight.

Day 9

9. Check colonies on the LB+Cm+Strep plates. There will likely be many colonies on both the "mutation oligo" plate and the "no DNA" plate. Set up 24 2ml microfuge tubes with 1.9 ml LB+Cm+Strep and inoculate single colonies from the LB+Cm+Strep plate. Inoculate cultures with the original BAC and the cassette integration as controls. Grow overnight at 37 °C, shaking at 1000 rpm

Day 10

10. Check for replacement of the cassette with the oligo by colony PCR on candidate bacteria. Use the same PCR reaction and program as when checking for cassette integration. Depending on the BAC DNA sequence, 20-100% of the candidates should now give PCR product size consistent with replacement of the cassette with the mutation.

11. In parallel to the colony PCR, save 30 µl of culture as a backup and use the rest of the culture for BAC DNA mini-preparation followed by restriction analysis (Hofemeister et al. 2010).

12. PCR products from correct integrations can now by purified and sequenced to verify the presence of the desired mutation.

Supplementary Protocol 2. Generating stable clonal cell line clones from BAC transgenes for molecular genetic analysis

Considerations

For genetic analysis, it is best to generate RNAi-resistant transenes so that the endogenous protein may be selectively depleted. Ideally, a 21-mer target sequence of a characterized siRNA can be mutated at amino acid wobble positions. Alternatively, BACs containing a homologous gene of interest from an organism different than that being transfected may be used. In this case, it is possible that the nucleotide sequence may be different enough to convey resistance.

There are many methods and reagents available for transfection of DNA into cell lines. Many of these will work for BAC DNA, with the appropriate optimization. Similarly, most cell lines that can be transfected with plasmid DNA can be transfected with BAC DNA, with appropriate optimization and techniques (with significantly lower efficiency). We most routinely use Effectene transfection reagent (Qiagen) for transfecting HeLa and U2OS cell lines, as detailed in the protocol below.

Purification of BAC DNA for transfection

1. Grow 50ml cultures of bacteria containing the BAC(s) of interest in LB containing appropriate antibiotics to OD600 ~1.8-2.0

2. Isolate BAC DNA following the protocol "Low-copy plasmid purification: Maxi/BAC" of the Nucleobond AX 100 kit. Check for BAC DNA on an agarose gel. Isolated BAC can be stored at 4°C for several weeks.

Transfecting Cell lines with a BAC construct

Materials

Hela/U2OS culture media: DMEM (Invitrogen), supplemented with 10% FBS, 100 units/ml Penicillin, and 100 µg/ml Streptomycin (Gibco)
G418 (Geneticin) (Invitrogen)
PBS
Effectine (Qiagen) transfection reagent (or other)

OptiMEM I (Invitrogen)

1. Transfect cells with Effectine as described in manufaturer's protocol. (other transfection methods may also be used). The optimal transfection reagents and quantities will differ with different cell lines. Below are the quantities used for U2OS and HeLa cells:

	Estimated BAC DNA	'Enhancer'	'Effectine'
U2OS	1ug (8-25ul from BAC prep)	6ul	20ul
HeLa	300ng (3-10ul from BAC prep)	8ul	10ul

2. The day after transfection, split cells and add G418 (or appropriate selection antibiotic) to media. For U2OS and HeLa cells, 400ug/ml G418 is used. Continue with selection until clear colonies are visible (hundreds of cells per colony; ~2 weeks).

3. Pick individual colonies from plates to isolate clonal cell lines (For genetic analysis of mutations it is necessary to have clonal cell lines) and transfer them to individual wells of 24-well plates. To begin, pick at least 20-30 colonies to grow up into individual clonal cell lines. Clones may also be isolated by single cell isolation using a FACS machine from a G418-resistant "pool" of transfected cells. However, this will add weeks to the procedure. Unpicked colonies left on plates may be pooled and frozen for later analysis or cell sorting.

4. Grow up clones over the next several days; transfer to 6-well plates to expand.

Analyzing and selecting clonal cell lines for analysis

This protocol assumes mutations are generated in an RNAi-resistant background for study. For accurate genetic analysis of mutations, cell lines expressing the BAC-transgene at near-endogenous levels is desired. Below is a typical workflow for identifying fluorescent protein-tagged clones to use for further analysis.

1. Screen through live cells with a fluorescence microscope to identify expressing clones.
If the cellular localization of the protein is known, correct localization of the wild type transgenic protein can be verified. Fluorescence levels can also be quantified as a measure of protein expression levels. For wild-type tagged proteins, clones containing incorrect fluorescence localizations can be discarded at this step.

2. Check relative protein levels in clones by Western blot. If an antibody to the tagged protein is available, protein levels relative to the endogenous protein can be judged. If not, antibodies against the protein tag may be used to observe relative expression levels between clones. In general, fluorescence intensity of a tagged protein correlates very well to protein levels by western.

3. Confirm RNAi-resistance of wild-type transgenes and functional rescue. Deplete endogenous protein by RNAi. If an antibody against the protein of interest is available, Western blots should confirm resistance of the transgenic protein to RNAi depletion. At the same time, functional assays can be performed after RNAi in original untagged cell lines and wild-type tagged protein clones to confirm functional rescue of the tagged transgene (and specificity of RNAi phenotype). If endogenous protein levels cannot be monitored, identify clones that rescue RNAi depletion functionally and have the lowest expression levels of the transgenic protein. From these analyses, identify a few clonal cell lines expressing wild-type transgenic protein to serve as controls for analysis of mutant protein function.

4. Identify mutant-tagged protein cell clones that express the protein at similar levels to the selected wild-type clones, and are also RNAi-resistant by western blot. Use more than one clone of each mutation for subsequent functional/phenotypic analyses to rule out clonal differences (i.e. integration site differences, genome differences) between cell lines.