Recombineering Manual

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BAC Transformation

BACs may be transformed into either DY380, EL250 or EL350 cells. Transformation requires high quality BAC DNA. Purification using a commercial kit designed for BAC isolation is highly recommended.

BAC DNA Purification

Prepare BAC DNA following manufacturers directions.

DO NOT completely dry BAC pellet or it will never fully resuspend.

DO NOT vortex the resuspended BAC DNA.

AVOID repeated pipeting and use a LARGE BORE tip when pipeting BAC DNA to avoid shearing.

ALIQUOT and FREEZE BAC DNA at -20°C. BAC DNA stored at 4°C rapidly degrades and will be unsuitable for transformation after even a few days. Also avoid repeated freeze-thaw cycles.

TRANSFORMATION

Prepare Electrocompetent Cells

1. Grow a 5 ml culture of DY380 in LB-Lennox O/N at 32°C.

2. Prechill sterile ddH$_2$O and 50 ml conical tube on ice. Cool centrifuge to 0°C and microcentrifuge to 4ºC.

3. Use 1 ml of O/N culture to start a culture in 50 ml of LB-Lennox in a 250 ml flask. A 50 ml culture will yield 5 × 50 μl aliqouts of electrocompetent cells, good for 5 transformations. Scale up as needed.

4. Grow the 50 ml culture at 32°C with shaking (225 rpm) until $OD_{600} \approx 0.6$. This usually takes 2 - 3 hours.

5. Immediately plunge the flask into an ice bath and swirl by hand until ice cold (≈ 5 min).

6. Transfer to a prechilled 50 ml conical tube and centrifuge at 1900xg 0°C for 15 min. If your centrifuge permits, RCF of up to 5000xg may be used.

7. Resuspend with 1 ml ice cold H_2O by pipeting back and forth. Then add 40 ml of ice cold H_2O and gently agitate. DO NOT VORTEX. Centrifuge for 15 min at 0ºC.

8. Repeat wash 2 more times for a total of 3 washes. CAUTION: As washes progress pellet will become looser.

9. Transfer to chilled microcentrifuge tube. Spin down at < 10,000 rpm 4°C briefly (30 sec).

10. Resuspend in a total volume of 250 µl ice cold H_2O and split into 5 x 50 µl aliqouts. Keep on ice until ready to be used. If desired, the electrocompetent cells may be resuspended in 10% glycerol in H_2O and stored at –80°C for a few days.

Electroporate BAC

1. Prechill 1 mm cuvettes BTX610.

2. Dilute BAC to 200 ng/µl and 1 µg/µl. This may be difficult since BAC prep is very viscous. DO NOT VORTEX.

3. To each 50 µl of electrocompetent cells, add 1 µl (200 ng – 1 µg) of BAC DNA. Mix by gently tapping the tube and keep on ice. Also transform 1 ng of some generic plasmid as a positive control for electroporation. It is recommended that a duplicate transformation be done since arcing could occur during electroporation.

4. Transfer transformation mix to cuvette, tap down on benchtop, and electroporate in BTX ECM630: 1750V, 200Ω, 25µF.

5. Recover with 1 ml LB and place in a 2 ml microcentrifuge tube. Shake horizontally at 32ºC for > 1 hour.

6. Plate on appropriate antibiotic plates. Grow at 32ºC. The number of colonies can vary greatly depending on the BAC. Pick colonies even if there is only one!

BAC minipreps

1. Pick colonies and grow 5 ml O/N cultures at 32ºC in appropriate antibiotic.

2. Transfer 4.5 ml to 15 ml conical tube and centrifuge 1900xg 0°C for 15 min. If your centrifuge permits, RCF of up to 5000xg may be used. Keep 0.5 ml of remaining culture for a glycerol stock.

3. Aspirate and resuspend in 250 µl Qiagen Buffer P1.

4. Add 250 µl Qiagen Buffer P2, mix by gentle inversion, and incubate at room temp for < 5 min.

5. Add 250 µl Qiagen Buffer N3, mix by gentle inversion, and incubate on ice for 5 min.

6. Spin max speed in microcentrifuge for 5 min and transfer supernatant to new tube.

7. Repeat step 6.

8. Add 750 µl 100% EtOH and gently mix. Incubate on ice 10 min.

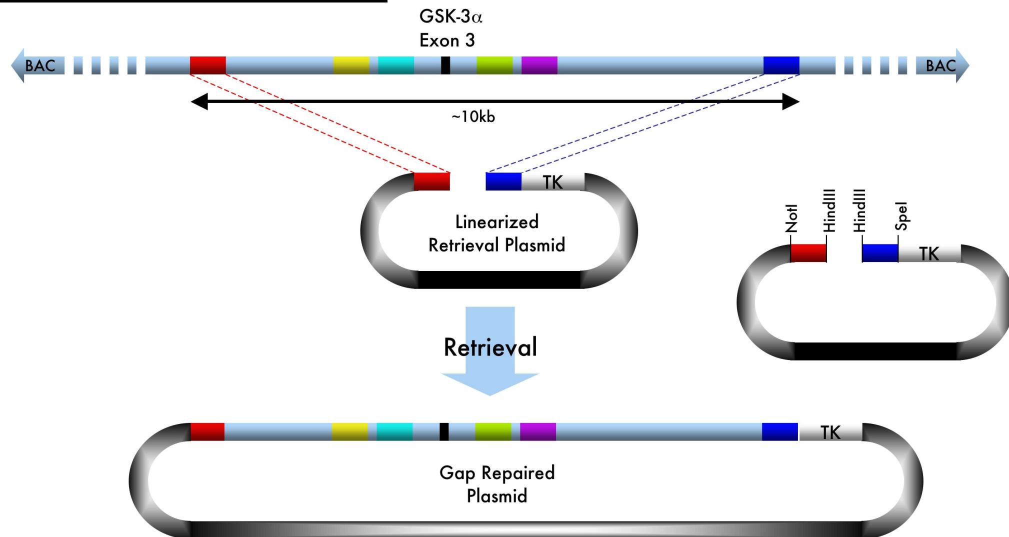
9. Spin max speed in microcentrifuge for 10 min. Aspirate.

10. Wash once with 1 ml 70% EtOH. Spin. Aspirate. Air dry very briefly. It is better to have the pellet still a little wet than too dry or it will never resuspend.

11. Resuspend in 50 µl TE. DO NOT VORTEX. Let pellet hydrate O/N at 4ºC.

12. Confirm BAC transformation by PCR and Restriction digest and compare to original BAC prep. The restriction digest is essential since BAC rearrangements can happen during transformation. PCR will not likely reveal such a rearrangement. These arrangements happen quite frequently.

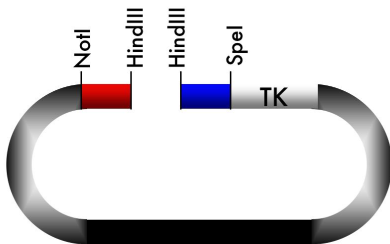
Construct Retrieval Plasmid



Primer Design

Select a 10 – 15 kb region from the BAC that contains the locus to be floxed. The PCR primers should amplify 300 – 500 bp homology arms (blue & red boxes) at the ends of the fragment to be retrieved.

5'Retrieval-F NotI	ATAAGCGGCCGCXXXXXXXXXXXXXXXXXXXXXX
5'Retrieval-R HindIII	GTCAAGCTTXXXXXXXXXXXXXXXXXXXXXX
3'Retrieval-F HindIII	GTCAAGCTTXXXXXXXXXXXXXXXXXXXXXX
3'Retrieval-R SpeI	TCTACTAGTXXXXXXXXXXXXXXXXXXXXXX



Vector Construction

1. Amplify the homology arms by PCR amplification off of the BAC template.

1 µl BAC (4 ng/µl)
0.5 µl Forward primer
0.5 µl Reverse primer
2 µl 5mM dNTPs
5 µl 10X Thermo Pol Buffer (NEB)
0.2 µl Taq (NEB)
40.8 µl H_2O
—--------
50 µl

10'	95ºC	
1'	95ºC	X 35
1'	56ºC	X 35
1'	72ºC	X 35
10'	72ºC	

2. Gel purify PCR products using a gel extraction kit. It may become necessary to do multiple amplifications, run in multiple lanes on a gel, and combine into a single spin column to increase yield. If enough DNA is purified it can then be ethanol precipitated.

3. Digest the 5' homology arm with NotI and HindIII, and the 3' homology arm with SpeI and HindIII.

4. Purify with a spin column kit.

5. Digest pL253 with SpeI and NotI, and gel purify with a gel extraction kit.

6. Treat the linearized gel purified vector with Antarctic Phosphatase.

7. Ligate both homology arms to pL253 using T4 DNA ligase. Use ~100 ng of vector and ~25 ng of each homology arm for a 3:1 molar ratio. Include a vector only ligation as a negative control. Ligate 16ºC O/N.

8. Transform into competent cells (DH5α works fine). Plate on LB-Ampicillin and incubate 37°C O/N.

9. Do mini-preps and confirm by restriction digest.

Retrieve BAC

Linearize Retrieval plasmid

N.B. Retrieval plasmid must be fully digested prior to transformation. Even a small amount of undigested, circular plasmid will contribute to a high background.

1. Linearize retrieval plasmid by digesting with HindIII.

6 µl Retrieval plasmid (0.5 – 1 µg/µl)
18 µl 10X Buffer 2 (NEB)
144 µl H_2O
12 µl HindIII (20 u/µl NEB)
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180 µl

2. 37ºC O/N (>14 hrs)

3. Load 6 lanes on a 0.6% agarose gel (30 µl per lane)

4. Run 150 V for 5 hrs, cut out one large gel slice and put into a 15 ml conical tube.

5. Purify with a gel extraction kit (split dissolved agarose between 4 columns) and elute with 50 µl EB per column.

6. Combine together in a microcentrifuge tube, add 20 µl 3M Sodium Acetate and 500 µl 100% EtOH. Vortex and put on dry ice 10 min or -20ºC O/N. Centrifuge 10 min max speed at 4ºC. Wash 70% EtOH. Dry. Resuspend in 20 µl ddH_2O and obtain A_{260} with spectrophotometer.

Prepare Electrocompetent cells

N.B. Induction of λRED will require a 42ºC shaking water bath.

The cells grown at 32ºC will serve as a negative control for retrieval.

1. Grow a 5 ml culture of DY380 containing the BAC to be retrieved in LB-Lennox with chloramphenicol O/N at 32°C.

2. Prechill sterile ddH_2O on ice. Cool centrifuge to 0°C and microcentrifuge to 4ºC. Heat a shaking water bath to 42ºC.

3. Use 1 ml of O/N culture to start a culture in 50 ml of LB-Lennox in a 250 ml flask. This will yield 2 × 50 µl aliqouts of induced and 2 × 50 µl aliqouts of uniduced electrocompetent cells, good for 2 transformations. Scale up as needed.

4. Grow the 50 ml culture at 32°C with shaking (225 rpm) until $OD_{600} \approx 0.6$. This usually takes 2 - 3 hours.

5. Transfer two 20 ml aliquots into individual 50 ml conical tubes. Mark one "32" and the other "42". Incubate "32" in a 32ºC shaker for 15 min. Incubate "42" in a 42ºC <u>shaking water bath</u> for 15 min.

6. Immediately place both tubes in an ice water bath and rapidly cool by hand, stirring for 5 min.

7. Centrifuge at 1900xg 0°C for 15 min. If your centrifuge permits, RCF of up to 5000xg may be used.

8. Resuspend with 1 ml ice cold H_2O by pipeting back and forth. Then add 40 ml of ice cold H_2O and gently agitate. DO NOT VORTEX. Centrifuge for 15 min at 0ºC.

9. Repeat wash 2 more times for a total of 3 washes. CAUTION: As washes progress pellet will become looser.

10. Transfer to chilled microcentrifuge tubes. Spin down at < 10,000 rpm 4°C briefly (30 sec)

11. Resuspend each in a total volume of 100 µl ice cold H_2O and split into 2 x 50 µl aliqouts. Keep on ice until ready to be used. If desired the electrocompetent cells may be resuspended in 10% glycerol in H2O and stored at –80°C for a few days.

Electroporate Retrieval plasmid

1. Prechill 1 mm cuvettes BTX610.

2. Pipet 150 ng of linearized retrieval plasmid into an aliquot of electrocompetent cells. It is recommended that a duplicate transformation be done for both the 32 and 42, since arcing could occur during electroporation.

3. Mix by gently tapping the tube and keep on ice.

4. Transfer transformation mix to cuvette, tap down on benchtop, and electroporate in BTX ECM630: 1750V, 200Ω, 25μF.

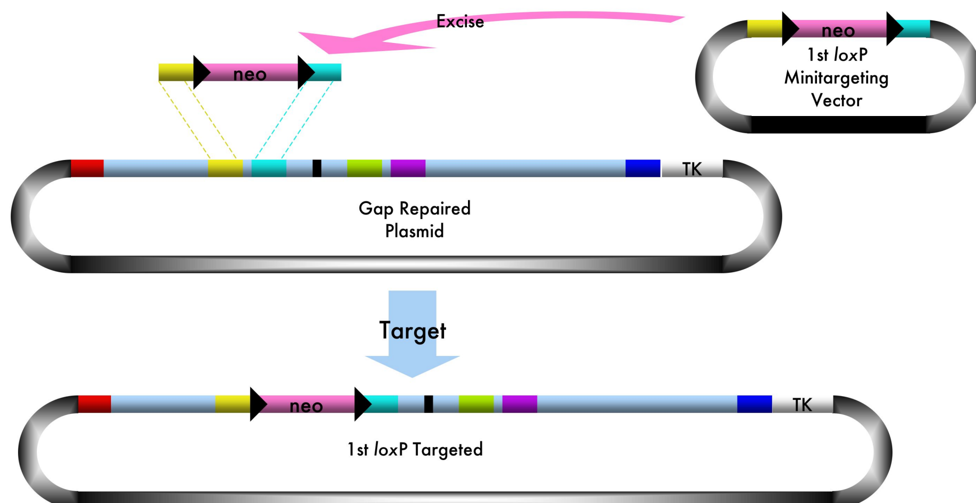
5. Recover with 1 ml LB and place in a 2 ml microcentrifuge tube. Shake horizontally at 32ºC for > 1 hour.

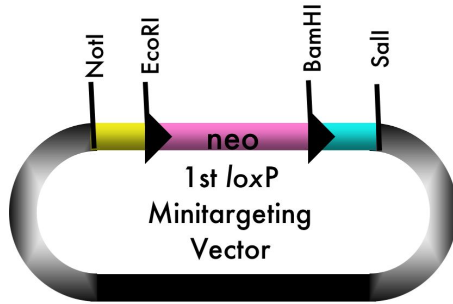
6. Plate on LB-Ampicillin plates. Grow at 32ºC. Should obtain many colonies from 42 and few from 32.

7. Do plasmid mini-preps and confirm by restriction digest.

8. Make glycerol stocks of positive clones and do a large scale plasmid prep.

Construct 1st Mini-targeting Vector





Primer Design

As with the retrieval homology arms, PCR primers should amplify 300 – 500 bp products. The number of bp between the two homology arms should be minimal. It is also wise to include an additional restriction site in one of these primers (highlighted in green). Ideally, this restriction site should not be present in the retrieved sequence but just outside of the retrieved sequence. This is helpful for genotyping ES cells by Southern blot.

5'-1target-F NotI	ATAAGCGGCCGCXXXXXXXXXXXXXXXXXXXXX
5'-1target-R EcoRI	GTCGAATTCNNNNNNXXXXXXXXXXXXXXXXXXXXX
3'-1target-F BamHI	ATAGGATCCXXXXXXXXXXXXXXXXXXXXX
3'-1target-R SalI	GTCGTCGACXXXXXXXXXXXXXXXXXXXXX

Vector Construction

1. Amplify the homology arms by PCR amplification off of the BAC template

1 µl BAC (4 ng/µl)
0.5 µl Forward primer
0.5 µl Reverse primer
2 µl 5mM dNTPs
5 µl 10X Thermo Pol Buffer (NEB)
0.2 µl Taq (NEB)
40.8 µl H_2O
—--------
50 µl

10'	95ºC	
1'	95ºC	X 35
1'	56ºC	X 35
1'	72ºC	X 35
10'	72ºC	

2. Gel purify PCR products using a gel extraction kit. It may become necessary to do multiple amplifications, run in multiple lanes on gel, and combine into a single spin column to increase yield. If enough DNA is purified it can then be ethanol precipitated.

3. Digest 5' homology arm with NotI and EcoRI, and the 3' homology arm with BamHI and SalI.

4. Purify with a spin column kit.

5. Digest *Neo lox*P cassette out of pL452 BamHI and EcoRI. Digest pBluescript (KS or SK +/-) with NotI and SalI. Gel purify the 1890 bp *Neo lox*P cassette and the 3 kb pBluescript.

6. Treat the pBluescript with Phosphatase.

7. Ligate both homology arms to pBluescript using T4 DNA ligase. Use 100 ng of Bluescript, 190 ng of *Neo lox*P cassette and 40 ng of each homology arm for a 3:1 molar ratio of insert:vector. Include a vector only ligation as negative control. Ligate 16ºC O/N.

8. Transform into competent cells (DH5α works fine). Plate on LB-Ampicillin and incubate 37°C O/N.

9. Do plasmid mini-preps and confirm by restriction digest.

10. Make glycerol stocks of positive clones and do a large scale plasmid prep.

Target 1st *lox*P cassette into Gap repaired plasmid

Excise *Neo lox*P cassette

1. Digest with PvuI, NotI and SalI and gel purify cassette. PvuI will cut the Bluescript vector. The Bluescript vector is about the same size as the *Neo lox*P cassette plus homology arms (approx 2.9 kb), so it is necessary to fragment Bluescript to facillitate pure isolation of the cassette. If PvuI cuts within the homology arms, find another Restriction Enzyme.

2. Store -20ºC

Prepare Electrocompetent cells

N.B. Induction of λRED will require a 42ºC shaking water bath.

The cells grown at 32ºC will serve as a negative control for retrieval.

1. Grow a 5 ml culture of DY380 containing the gap repaired plasmid (from glycerol stock) in LB-Lennox with Ampicillin O/N at 32°C.

2. Prechill sterile ddH_2O on ice. Cool centrifuge to 0°C and microcentrifuge to 4ºC. Heat a shaking water bath to 42ºC.

3. Use 1 ml of O/N culture to start a culture in 50 ml of LB-Lennox in a 250 ml flask. This will yield 2 × 50 µl aliqouts of induced and 2 × 50 µl aliqouts of uniduced electrocompetent cells, good for 2 transformations. Scale up as needed.

4. Grow the 50 ml culture at 32°C with shaking (225 rpm) until OD_{600} ≈ 0.6. This usually takes 2 - 3 hours.

5. Transfer two 20 ml aliquots into individual 50 ml conical tubes. Mark one “32” and the other “42”. Incubate “32” in a 32ºC shaker for 15 min. Incubate “42” in a 42ºC shaking water bath for 15 min.

6. Immediately place both tubes in an ice water bath and rapidly cool by hand stirring for 5 min.

7. Centrifuge at 1900xg 0°C for 15 min. If your centrifuge permits, RCF of up to 5000xg may be used.

8. Resuspend with 1 ml ice cold H_2O by pipeting back and forth. Then add 40 ml of ice cold H_2O and gently agitate. DO NOT VORTEX. Centrifuge for 15 min at 0ºC.

9. Repeat wash 2 more times for a total of 3 washes. CAUTION: As washes progress pellet will become looser.

10. Transfer to chilled microcentrifuge tubes. Spin down at < 10,000 rpm 4°C briefly (30 sec)

11. Resuspend each in a total volume of 100 µl ice cold H_2O and split into 2 x 50 µl aliqouts. Keep on ice until ready to be used. If desired

the electrocompetent cells may be resuspended in 10% glycerol in H_2O and stored at –80°C for a few days.

Electroporate *Neo lox*P cassette

1. Prechill 1 mm cuvettes BTX610.

2. Pipet 25 ng of gel purified excised *Neo lox*P cassette into an aliquot of electrocompetent cells. It is recommended that a duplicate transformation be done for both the 32 and 42, since arcing could occur during electroporation.

3. Mix by gently tapping the tube and keep on ice.

4. Transfer transformation mix to cuvette, tap down on benchtop, and electroporate in BTX ECM630: 1750V, 200Ω, 25μF.

5. Recover with 1 ml LB and place in a 2 ml microcentrifuge tube. Shake horizontally at 32ºC for > 1 hour.

6. Plate on LB-Kanamycin plates. Grow at 32ºC. Should obtain many colonies from 42 and few from 32.

7. Do plasmid mini-preps and confirm by restriction digest.

8. Make glycerol stocks of positive clones.

Retransform gap repaired plasmid with 1st loxP cassette

N.B. This step is essential as there will be a mixed population of both targeted and untargeted gap repaired plasmid as long interlinked chains.

Use a mini-prep of a positive clone from the previous step (targeting).

1. Linearize the targeted plasmid with a Restriction Enzyme that cuts only once.

2. Religate with T4 DNA Ligase. Include a digested plasmid without Ligase as a negative control for the following transformation.

3. Transform ligation into chemically competent cells such as DH10B or One Shot Top10 Invitrogen.

4. Plate on to LB Kanamycin plates.

5. Do plasmid mini-preps and confirm by restriction digest.

6. Make glycerol stocks of positive clones.

Pop out *Neo* loxP cassette

N.B. This step is necessary in order to select for the second *Neo* loxP cassette which also requires Kanamycin selection.

Prepare Electrocompetent cells

1. Grow a 5 ml culture of EL350 in LB-Lennox O/N at 32°C.

2. Prechill sterile ddH_2O and 50 ml conical tube on ice. Cool centrifuge to 0°C and microcentrifuge to 4ºC.

3. Use 2 ml of O/N culture to start a culture in 50 ml of LB-Lennox in a 250 ml flask. 50 ml culture will yield 5 × 50 µl aliqouts of electrocompetent cells, good for 5 transformations. Scale up as needed.

4. Grow the 50 ml culture at 32°C with shaking (225 rpm) until $OD_{600} \approx 0.6$. This usually takes 2 - 3 hours.

5. Add 0.5 ml of 10% L-(+)-Arabinose and shake at 32°C for 1 hour.

6. Immediately plunge the flask into an ice bath and swirl by hand until cool (≈ 5 min).

7. Transfer to a prechilled 50 ml conical tube and centrifuge at 1900xg 0°C for 15 min. If your centrifuge permits, RCF of up to 5000xg may be used.

8. Resuspend with 1 ml ice cold H_2O by pipeting back and forth. Then add 40 ml of ice cold H_2O and gently agitate. DO NOT VORTEX. Centrifuge for 15 min at 0ºC.

9. Repeat wash 2 more times for a total of 3 washes. CAUTION: As washes progress pellet will become looser.

10. Transfer to chilled microcentrifuge tube. Spin down at < 10,000 rpm 4°C briefly (30 sec).

11. Resuspend in a total volume of 250 µl ice cold H_2O and split into 5 x 50 µl aliqouts. Keep on ice until ready to be used. If desired the electrocompetent cells may be resuspended in 10% glycerol in H2O and stored at –80°C for a few days.

Electroporate 1st targeted loxP plasmid into Cre induced cells

1. Prechill 1 mm cuvettes BTX610.

2. Pipet 1 ng of gap repaired plasmid with 1st loxP cassette retransformed into an aliquot of electrocompetent cells. It is recommended that a duplicate transformation be done since arcing could occur during electroporation.

3. Mix by gently tapping the tube and keep on ice.

4. Transfer transformation mix to cuvette, tap down on benchtop, and electroporate in BTX ECM630: 1750V, 200Ω, 25µF.

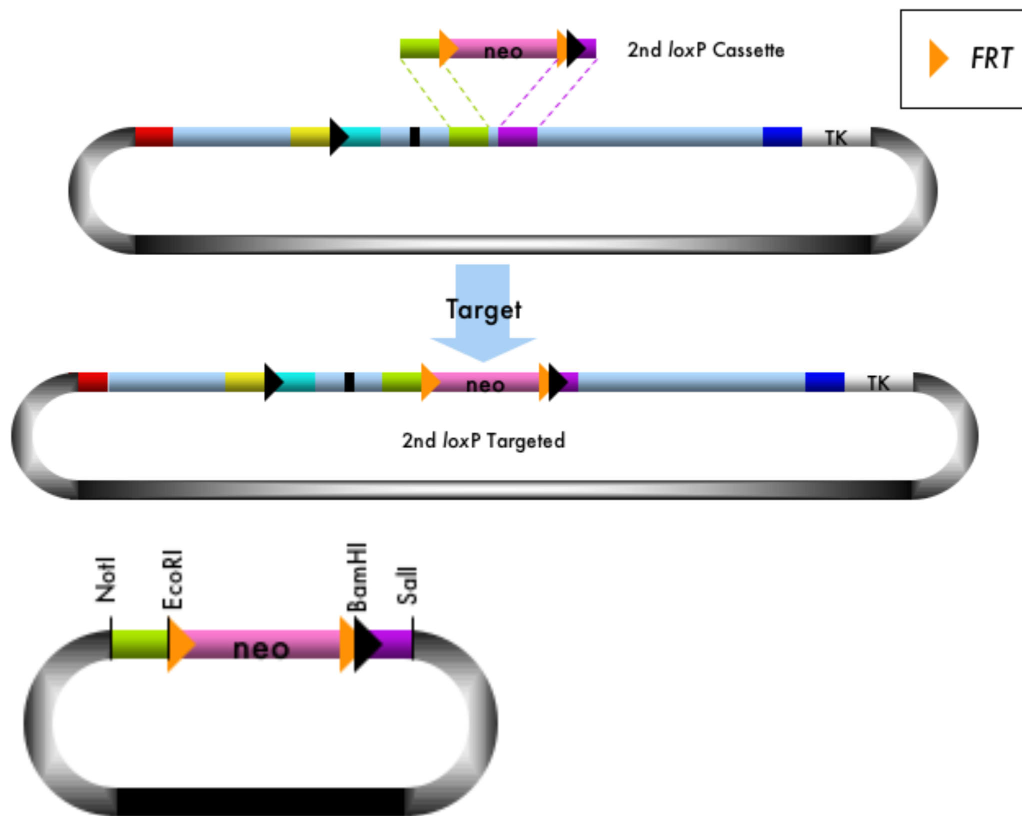
5. Recover with 1 ml LB and place in a 2 ml microcentrifuge tube. Shake horizontally at 32ºC for > 1 hour.

6. Plate on LB-Ampcillin plates. Grow at 32ºC.

7. Do plasmid mini-preps and confirm by restriction digest.

8. Make glycerol stocks of positive clones.

Construct 2nd Mini-targeting Vector



Primer Design

As with the retrieval homology arms, PCR primers should amplify 300 – 500 bp products. The number of bp between the two homology arms should be minimal. It is also wise to include an additional restriction site in one of these primers (highlighted in green). Ideally, this restriction site should not be present in the retrieved sequence but just outside of the retrieved sequence. This is helpful for genotyping ES cells by Southern blot.

5'-1target-F NotI ATAAGCGGCCGCXXXXXXXXXXXXXXXXXXXXX
5'-1target-R EcoRI GTCGAATTCNNNNNNXXXXXXXXXXXXXXXXXXXXX
3'-1target-F BamHI ATAGGATCCXXXXXXXXXXXXXXXXXXXXX
3'-1target-R SalI GTCGTCGACXXXXXXXXXXXXXXXXXXXXX

Vector Construction

1. Amplify the homology arms by PCR amplification off of the BAC template

1 µl BAC (4 ng/µl)
0.5 µl Forward primer
0.5 µl Reverse primer
2 µl 5mM dNTPs
5 µl 10X Thermo Pol Buffer (NEB)
0.2 µl Taq (NEB)
40.8 µl H_2O
—--------
50 µl

10’	95ºC	
1’	95ºC	X 35
1’	56ºC	X 35
1’	72ºC	X 35
10’	72ºC	

2. Gel purify PCR products using a gel extraction kit. It may become necessary to do multiple amplifications, run in multiple lanes on gel, and combine into a single spin column to increase yield. If enough DNA is purified it can then be ethanol precipitated.

3. Digest 5’ homology arm with NotI and EcoRI, and the 3’ homology arm with BamHI and SalI.

4. Purify with a spin column.

5. Digest *Neo lox*P cassette out of pL451 BamHI and EcoRI. Digest pBluescript (KS or SK +/-) with NotI and SalI. Gel purify the 1892 bp *Neo lox*P cassette and the 3 kb pBluescript.

6. Treat the pBluescript with Phosphatase.

7. Ligate both homology arms to pBluescript using T4 DNA ligase. Use 100 ng of Bluescript, 190 ng of *Neo lox*P cassette and 40 ng of each homology arm for a 3:1 molar ratio of insert:vector. Include a vector only ligation as negative control. Ligate 16ºC O/N.

8. Transform into competent cells (DH5α works fine). Plate on LB-Ampicillin and incubate 37°C O/N.

9. Do plasmid mini-preps and confirm by restriction digest.

10. Make glycerol stocks of positive clones and do a large scale plasmid prep.

Target 2nd *lox*P cassette into Gap repaired plasmid

Excise *Neo lox*P cassette

1. Digest with PvuI, NotI and SalI and gel purify cassette. PvuI will cut the Bluescript vector. The Bluescript vector is about the same size as the *Neo lox*P cassette plus homology arms (approx 2.9 kb), so it is necessary to fragment Bluescript to facillitate pure isolation of the cassette. If PvuI cuts within the homology arms, find another Restriction Enzyme.

2. Store -20ºC

Prepare Electrocompetent cells

N.B. Induction of λRED will require a 42ºC shaking water bath.

The cells grown at 32ºC will serve as a negative control for retrieval.

1. Grow a 5 ml culture of DY380 containing the gap repaired plasmid (from glycerol stock) in LB-Lennox with Ampicillin O/N at 32°C.

2. Prechill sterile ddH_2O on ice. Cool centrifuge to 0°C and microcentrifuge to 4ºC. Heat a shaking water bath to 42ºC.

3. Use 1 ml of O/N culture to start a culture in 50 ml of LB-Lennox in a 250 ml flask. This will yield 2 × 50 µl aliqouts of induced and 2 × 50 µl aliqouts of uniduced electrocompetent cells, good for 2 transformations. Scale up as needed.

4. Grow the 50 ml culture at 32°C with shaking (225 rpm) until $OD_{600} \approx 0.6$. This usually takes 2 - 3 hours.

5. Transfer two 20 ml aliquots into individual 50 ml conical tubes. Mark one “32” and the other “42”. Incubate “32” in a 32ºC shaker for 15 min. Incubate “42” in a 42ºC shaking water bath for 15 min.

6. Immediately place both tubes in an ice water bath and rapidly cool by hand stirring for 5 min.

7. Centrifuge at 1900xg 0°C for 15 min. If your centrifuge permits, RCF of up to 5000xg may be used.

8. Resuspend with 1 ml ice cold H_2O by pipeting back and forth. Then add 40 ml of ice cold H_2O and gently agitate. DO NOT VORTEX. Centrifuge for 15 min at 0ºC.

9. Repeat wash 2 more times for a total of 3 washes. CAUTION: As washes progress pellet will become looser.

10. Transfer to chilled microcentrifuge tubes. Spin down at < 10,000 rpm 4°C briefly (30 sec)

11. Resuspend each in a total volume of 100 µl ice cold H_2O and split into 2 x 50 µl aliqouts. Keep on ice until ready to be used. If desired the electrocompetent cells may be resuspended in 10% glycerol in H2O and stored at –80°C for a few days.

Electroporate *Neo lox*P cassette

1. Prechill 1 mm cuvettes BTX610.

2. Pipet 25 ng of gel purified excised *Neo lox*P cassette into an aliquot of electrocompetent cells. It is recommended that a duplicate transformation be done for both the 32 and 42, since arcing could occur during electroporation.

3. Mix by gently tapping the tube and keep on ice.

4. Transfer transformation mix to cuvette, tap down on benchtop, and electroporate in BTX ECM630: 1750V, 200Ω, 25µF.

5. Recover with 1 ml LB and place in a 2 ml microcentrifuge tube. Shake horizontally at 32ºC for > 1 hour.

6. Plate on LB-Kanamycin plates. Grow at 32ºC. Should obtain many colonies from 42 and few from 32.

7. Do plasmid mini-preps and confirm by restriction digest.

8. Make glycerol stocks of positive clones.

Retransform gap repaired plasmid with 2nd loxP cassette

N.B. This step is essential as there will be a mixed population of both targeted and untargeted gap repaired plasmid

Use a mini-prep of a positive clone from the previous step.

1. Linearize the targeted plasmid with a Restriction Enzyme that cuts only once.

2. Religate with T4 DNA Ligase. Include a digested plasmid without Ligase as a negative control for the following transformation.

3. Transform ligation into chemically competent cells such as DH10B or One Shot Top10 Invitrogen.

4. Plate on to LB Kanamycin plates.

5. Do plasmid mini-preps and confirm by restriction digest.

6. Make glycerol stocks of positive clones.

7. Should be ready for targeting into mouse ES cells.

Test ability to excise with Cre and Flp

EL250 & EL350 can be induced by arabinose to test the functionality of *lox*P and FRT sites. Follow the procedure outlined in the section titled 'Pop out *Neo* loxP cassette'.

Strains

DY380	temp sensitive λ*RED*, *tet*^{R}
EL250	temp sensitive λ*RED*, Arabinose inducible *flpe*
EL350	temp sensitive λ*RED*, Arabinose inducible *cre*

Plasmids

PL451	FRT-*Neo*-FRT-*lox*P
PL452	*lox*P-*Neo*-*lox*P

LB Lennox

10 g Tryptone
5 g Yeast Extract
5 g Sodium Chloride

up to 1 L dd$H_2$0

Antibiotic working concentrations		Stock	
Ampicillin	100 µg/ml	50 mg/ml	in ddH_2O
Kanamycin	25 µg/ml	10 mg/ml	in ddH_2O
Tetracycline	12.5 µg/ml	12.5 mg/ml	in 70% EtOH
Chloramphenicol	12.5 µg/ml	12.5 mg/ml	in MeOH