Generation of Gene Targeting Vectors using Recombineering

- **Small scale (single tube) protocol**

Reagent information

All TSA bacterial liquid cultures are grown in:

1xTerrific Broth (TB) supplemented with 0.4% Glycerol and the appropriate antibiotics:

For 1L
11.8 g Bacto-tryptone
23.6 g yeast extract
9.4 g dipotassium hydrogen phosphate (anhydrous)
2.2 g potassium dihydrogen phosphate (anhydrous)
0.4% glycerol (added after autoclaving)

Alternatively, use Invitrogen Terrific Broth 2211-022. Dissolve 47gr powder in 1L dH20 and add 4ml glycerol before autoclaving.

Antibiotics

Kanamycin stock solution: 10 mg/ml, final concentration 30 µg/ml
Tetracyclin stock solution: 5 mg/ml, final concentration 5 µg/ml
Zeocin stock solution: 10 mg/ml, final concentration 50 µg/ml

- All water used is HPLC grade.
- All reagents are purchased from Sigma-Aldrich, unless otherwise indicated.
- Primers are ordered from Invitrogen. We receive them at 100uM concentration:
    - Working stocks are 2 µM prepared with HPLC water.
- Biorad Gene Pulser X cell is used for transformations
- 2X GoTaq Green master mix is from Promega Cat no: M7113
- Advantage2 polymerase kit is from Clontech. Cat no: 639202
- DpnI is from New England Biolabs
- Zeocin is bought in powder form from Source Bioscience (Cat no: ant-zn-1p) 100mg/ml stock is prepared by dissolving 0.1gr Zeocin in 1ml of HPLC water. It is filtered with 0.2u filter and stored at -20^{o}C in 100 µL aliquots. Dilute 10X for 10mg/ml stock.
- Yeg-Cl plates made with 5g Yeast Extract, 5g NaCl, 2g 4-Chloro-DL-phenylalanine, 15g Agar and 0.4% glucose with 30µg /ml kan.
- 14ml Greiner tubes are used.

- Dialysis filters are from Millipore Cat no: VCWP02500
- 10% L-Arabinose stock is prepared by dissolving 1gr L-Arabinose in 10ml of HPLC water. It is filtered with 0.2u filter and stored at 4°C in aliquots.
- Gateway® LR Clonase® enzyme mix 80ul is from Invitrogen Cat no: 11791-019

Hazards & Personal Protective Equipment

- Microbiological agents
- Moving parts (centrifuge)
- Electricity (electroporation system, centrifuge, water bath)
- Repetitive movements (pipettes)

Course participants are required to wear laboratory coats and gloves at all time during the practical.

Steps *in italics* will be done for you during the course

Day 0

PbG library clone glycerols are verified for the presence of your gene by PCR.

1. *Set up overnight culture of PbG clone. Inoculate from glycerol stock to a 14 ml Greiner tube containing 4.0 ml TB medium containing 30 µg/ml kanamycin (12 µl) to select for the pJazz vector. Use a loosely fitting cap to allow air into the tube. Shake overnight at 37°C.*

Day 1

Before you start: Chill ddH2O and electroporation cuvettes on ice for > 2h and cool benchtop centrifuge to 4°C.

1. *Check OD600 o/n cultures at a 1/10 dilution by diluting 100 µL bacterial culture in 900 µL TB and using a cell-density meter.*
2. *Dilute the cultures to a final OD_{600nm} of 0.05:*
    a. *Add 4 mL TB medium, with 0.4% glycerol and 30µg/ml kanamycin (12 µl) in a 14 ml Greiner tube.*
    b. *Calculate dilution factor: 4000 µL / (Average culture OD600 / 0.05) = Culture inoculum volume.*
    c. *Add the appropriate volume o/n culture to the Greiner tube.*
3. *Incubate at **37°C** shaking at 225 rpm.*
4. *Grow to an OD_{600} of 0.6-0.8 (Approx. 2-2½ h).*

START PRACTICAL DAY 1

5. Chill tube on ice for 15 min and then transfer 1.4 ml of culture to 1.5 ml centrifuge tube. Spin 3 min at 5,000 g in a cold microfuge. Keep cold from this point onwards!

6. Resuspend the cell pellet in 1ml ice cold ddH2O.

7. Spin 3 min at 5,000 g in a cold microfuge. Carefully aspirate supernatant.

8. Repeat 2x more washes with cold ddH2O. The cell pellet becomes looser with each subsequent wash. Make sure cells are drained well after final wash.

9. Keeping the tube on ice, resuspend cells in 50 µl cold ddH2O, add 2 µl (10 ng total, 5ng/ µl) of pSC101gbdA recombinase plasmid, and transfer to a chilled 1 mm gap electroporation cuvette.

10. Electroporate at 1800 V, 10 µF, 600 Ω using Biorad Gene Pulser Xcell.

11. Add 950 µl of recovery medium (TB medium + 0.4% glycerol without antibiotics, transfer to a 14 ml Greiner tube and incubate shaking at **30°C** for 70 min.

    NOTE: DO NOT CULTURE AT 37°C.

12. *Add 3 ml 1 x TB medium with 0.4% glycerol, containing kanamycin (12 µl of 10 mg/ml stock) and tetracycline (4 µl of 5 mg/ml stock) and shake over night at **30°C**.*

    NOTE: DO NOT CULTURE AT 37°C.

Generation of Zeo/PheS PCR product with recombineering homology arms

H2O	*15.5 µl*
pR6K attR1-zeo/pheS-attR2 plasmid template (12 ng/µl)	*1.0 µl*
10x buffer	*2.5 µl*
Primer R1 with target-specific extensions (2 µM)	*2.5 µl*
Primer R2 with target-specific extensions (2 µM)	*2.5 µl*
dNTP mix (10 mM each)	*0.5 µl*
AdvantageTaq2 (Clontech)	*0.5 µl*
Total	*25.0 µl*

PCR conditions:

95°C 5' // 95°C 30'' / 58°C 30'' / 72°C 1'30'' (x30) // 72°C 10' // 4°C hold

13. *Check 2.5 µl of PCR product on gel. Expected size is 1.8kb*

14. *Add 1 µl DpnI (stock is 20 U/µl) directly and incubate for 1 h at 37°C.*

15. *Dialyze with 0.1 µM pore filters from Millipore against water for 1 h.*

16. *Determine DNA concentration by using spectrophotometer. Store at* ***-20°C***

Day 2

Before you start: Chill ddH_2O and electroporation cuvettes on ice and cool benchtop centrifµge to 4°C.

1. *Check OD600 o/n cultures at a 1/10 dilution by diluting 100 µL bacterial culture in 900 µL TB and using a cell-density meter.*
2. *Dilute the cultures to a final OD_{600nm} of 0.05:*
    a. *Add 4 mL TB medium, with 0.4% glycerol and 30µg/ml kanamycin (15 µl), 5 µg/ml tetracycline (5 µl) in 14 ml Greiner tube.*
    b. *Calculate dilution factor: 4000 µL / (Average culture OD600 / 0.05) = Culture inoculum volume.*
    c. *Add the appropriate volume o/n culture to the Greiner tube*

3. *Incubate at **30°C** shaking at 225 rpm.*

    NOTE: DO NOT CULTURE AT 37°C AT THIS STAGE.

4. *Grow to an OD_{600} of 0.2-0.4 (Approx. 2-2½ h), and induced the expression of recombinase genes by adding 80 µl 10% L-arabinose and transfer to:*
    a. ***37°C** stationary water bath for 5 min.*
    b. ***37°C** shaking incubator for 35 min.*

START PRACTICAL DAY 2

5. Chill tube on ice for 15 min and transfer 1.4 ml of culture to 1.5 ml centrifuge tube. Spin 3 min at 5,000 g in a cold microfuge. Keep cold from this point onwards!

6. Resuspend the cell pellet in 1ml ice cold ddH2O.

7. Spin 3 min at 5,000 g in a cold microfuge. Carefully aspirate supernatant.

8. Repeat 2 more washes with cold ddH2O. The cell pellet becomes looser with each subsequent wash. Make sure cells are drained well after final wash.

9. Keeping the tube on ice, resuspend cells with 250ng-1µg of dialysed PCR product in 50µl cold ddH2O and transfer to a chilled 1 mm electroporation cuvette.

10. Electroporate at 1800 V, 10 µF, 600 Ω using Biorad Gene Pulser Xcell.

11. Add 950 µl of recovery medium (TB medium) + 0.4% glycerol without antibiotics, transfer to a 14 ml Greiner tube and shake at **37°C** for 70 min.

12. *Add 3 ml 1 x TB medium with 0.4% glycerol, containing zeocin (20 µl of 10 mg/ml stock) and kanamycin (12 µl of 10 mg/ml stock), and shake 16hrs at **37°C.***

While establishing the recombineering method in your lab it is highly advisable you streak out cells on zeocin and kanamycin selective plates at this stage and screen single colonies for evidence of successful recombineering. To help you trouble shoot, carry along controls in which you do not add PCR product, omit arabinose induction, or omit the recombinase plasmid. Negative controls should not produce colonies.

You may genotype single colonies by PCR to check the desired recombination product is present and to estimate how many colonies that still contain some unrecombined plasmid. Miniprep DNA from single colonies may be retransformed into TSA cells to help eliminate any background of unrecombined ("wt") plasmid.

Day 3

START PRACTICAL DAY 3

1. Using the Qiagen Miniprep procedure and buffers isolate pJAZZ DNA:
   Pellet 2ml of the culture at 13.000 rpm for 1 min at RT.Discard supernatant.
   a. Re-suspend the bacterial pellet in **500 µl** of buffer **P1** (ensuring that the buffer used has confirmation of the addition of RNase) and transfer to a 2ml eppendorf tube.
   b. Add **500 µl** of buffer **P2** (check to ensure no precipitation of the buffer has occurred) and gently invert 4-6 times, do not vortex the tubes, allow the lysis reaction to proceed 5 minutes. Not longer.
   c. Add **700 µl** of buffer **N3** and mix immediately by inverting the tube 4-6 times.
   d. Spin at 13000rpm for 10mins in a microcentrifµge.
   e. Pipette half of the supernatant ~800µl into the QIAprep spin column and spin at 13000rpm for 30s.
   f. Discard the flow through and pour the remaining supernatant into the spin column, spin at 13000rpm for 30s.
   g. Discard the flow through, add **500 µl** of buffer **PB** to the column and spin at 13000rpm for 30s.
   h. Discard the flow through, add **750µl** of buffer **PE** to the column and spin at 13000rpm for 30s.
   i. Discard the flow through and spin at 13000rpm for 60s to remove any residual wash buffer.
   j. Place the QIAprep column in a clean 1.5ml eppendorf tube, add **50 µl** of **TE** pH: 8.0 to the filter in the centre of the tube (avoid adding water to the edges of the tube where it does not come into contact with the filter), let the tube stand for 5 mins.
   k. Spin the tube at 13000 rpm for 1 min to elute the collected DNA.

2. Measure DNA concentration.

   When establishing the method it is advisable to run 10 µl pJazz DNA on 1% agarose gel to verify presence of your intermediate vector. Remember that pJazz vectors are low copy and the yield of your miniprep is going to be low.

3. Verify the recombineering reaction by running the PCR with following reagents and parameters:

Mini DNA	1 µl
H_2O	6.5 µl
Template (here lysate)	2.5 µl
2xGoTag Green master mix	12.5µl
QCR2 Primer (gene specific), (2µM)	2.5 µl
ZeoR2 Primer (generic), (2 µM)	2.5 µl
TOTAL	**25 µl**

For both KO (R2R1) and TAG (R1R2) designs use the ZeoR2 primer together with the gene specific QCR2 primer.

PCR conditions are:

95°C 5’ // 95°C 30’’ / 50°C 30’’ / 68°C 1’ (x30) // 68°C 10’ // 4°C hold

4. Set up Gateway® LR Clonase reaction in 0.5 ml PCR tube:

Miniprep DNA of intermediate vector (30 ng/µl)	10.00 µl
pR6K 3xHA attL1-hdhfr/yFCU-attL2 (100 ng/µl)	1.00 µl
LR clonase buffer 5X	4.00 µl
LR clonase enzyme mix	2.00 µl
TE	3.00 µl
Total	**20.00 µl**

5. Incubate over night at **25°C** in a PCR machine.

Day 4

1. *Add 1.0 µl proteinase K (2 µg/µl) to the Clonase® reaction and incubate 10 min at* ***37°C.*** *Dialyse samples on Milipore membranes against water for 1h.*

2. *Dialyze with 0.1 µM pore filters from Millipore against water for 1 h.*

START PRACTICAL DAY 4

3. Put the required number of YEG-Cl Kan agar plates in a **37°C** incubator to warm up.

4. Thaw one vial of electrocompetent TSA cells on ice, 5 *µl* of the dialysed Clonase® reaction product and transfer to a chilled 1 mm gap electroporation cuvette.

5. Electroporate at 1800 V, 10 µF, 600 Ω using Biorad Gene Pulser Xcell.

6. Add 950 µl of recovery medium (TB medium + 0.4% glycerol) without antibiotics, transfer to round bottom tube (e. g. 10 ml Greiner) and shake at 37°C for 70 min.

6. *Transfer media from culture tubes to 1.5ml eppendorf tubes, spin for 2 minutes at 6000rpm.*

7. *Remove ~800µl of the supernatant then re-suspend cells in the remaining 200µl.*

8. *Add the 200µl of cells to the pre-warmed agar plates, spread the cells over the plate with a sterile spreader.*

9. *Incubate plates overnight at* ***37°C*** *to select for kanamycin resistant GW pJAZZ and against Zeo/PheS.*

Day 5

1. *Pick 4 colonies for PCR to verify correct clones.*

2. *Positive Gateway clones can be identified by PCR directly from the colonies with a gene specific primer and a primer annealing within the Gateway® cassette.*

3. *Pick a colony with a pipette tip:*
   a. *Dip in 4 ml 1 x TB with 0.4% glycerol with 30 µg/ml kanamycin to grow the colony o/n shaking at 37°C to generate liquid culture for glycerol stocks.*
      AND
   b. Dip into 10 µl of dH_20 and boil for 10 min at 95°C to use as PCR template.

4. *Prepare your PCR master mix:*

H_2O	5 µl
Template (colony lysate)	2.5 µl
2xGoTag Green master mix	12.5µl
QCR2 Primer (gene specific), (2µM)	2.5 µl
GW2 Primer (generic), (2 µM)	2.5 µl
TOTAL	**25 µl**

For both KO (R2R1) and TAG (R1R2) designs use the GW2 primer together with the gene specific QCR2 primer.

PCR conditions:
95°C 5' // 95°C 30'' / 50°C 30'' / 68°C 1' (x30) // 68°C 10' // 4°C hold

5. After verification, perform mini or midi prep followed by NotI digestion to continue with *P. berghei* transfection.