P(acman): Recombineering Fly

Koen Venken Modified 08-21-07

A. P(acman) plasmids fly

-**P(acman)-F-2**: conventional fly transgenic rescue construct
-**P(acman)-F-2-attB-2-4-2:** attB-plasmid of conventional fly transgenic rescue construct

-**P(acman)-F-2-Amp-5**: conventional fly transgenic rescue construct
-**P(acman)-F-2-Amp-5-attB-7-5-1:** attB-plasmid of conventional fly transgenic rescue construct

Used antibiotics:

-Cl12.5 (Chloramphenicol 12.5 µg/ml)
-T10 (Tetracyclin 10 µg/ml)
-A100 (Ampicillin 100 µg/ml)

B. Gap-repair protocol

Protocol for retrieval from BACs (Cl12.5) into A100 P(acman) plasmids. The same protocol holds for the Cl12.5 P(acman) plasmids, but other antibiotic combinations are necessary during certain steps. Gap-repair has been performed successfully from low copy plasmids such as BACs, Amp modified BACs, P1 bacteriophage; high copy plasmids such as pUAST. Conventional cloning through regular PCR has also been done.

B1. Primer design:
4 primersets are designed:
B1.A Conventional PCR for three-way ligation:
-Primerset for LA:
-goi-LA-AscI-F: aGGCGCGCC-goi
-goi-LA-BamHI-R: cgcGGATCC-goi
-Primerset for RA:
-goi-RA-BamHI-F: cgcGGATCC-goi
-goi-RA-PacI-R: accTTAATTAA-goi
-Primerset to test for triple ligation and sequencing over gap-repair junctions:
-goi-LA-Seq-F
-goi-RA-Seq-R
-Primerset to screen for correct gap-repaired clones
-goi-5'-check-R
-goi-3'-check-F

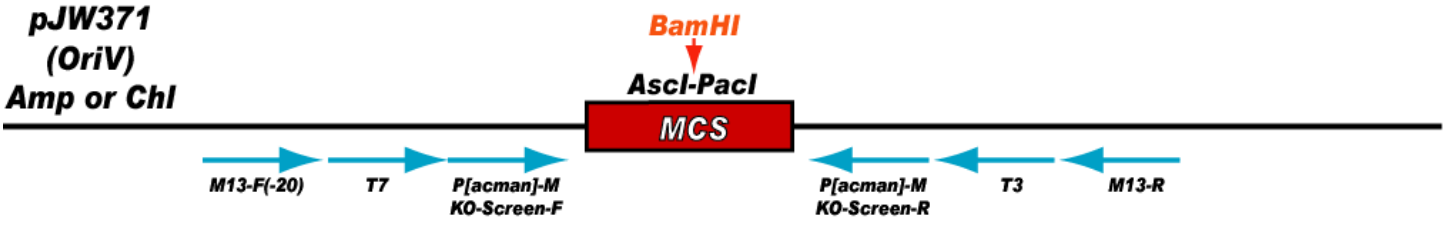

Fig. P(acman)-FLY: Multiple cloning site

Fig. Primersets

B1.B Two-round chimeric PCR for two-way ligation:

Similar principle as above, except that both PCRs for the LA and RA are sealed together in a second round of PCR (middle primers are overlapping).

B2. Induction of desired P(acman) construct in EPI300 cells for high-copy miniprep:

-Grow single colony in 1 ml ON at 37ºC in Falcon50 with A100.
-Add 9 ml with desired antibiotic (A100) and 10 µl CopyControl Solution (1000x Solution, Epicentre).
-Induce for 5 hrs.
-Do regular QIAGEN miniprep:
-Add 250 µl P1.
-Add 250 µl P2.
-Incubate at RT for 5'.
-Add 350 µl N3.
-Spin at 14.000 rpm, 10', RT.
-Load SN on column (QIAGEN).
-Spin briefly at 14.000 rpm, RT.
-Wash with 750 µl PE Buffer.
-Spin briefly at 14.000 rpm, RT.
-Spin at 14.000 rpm, RT, 1'.
-Elute with 50 µl EB (10 mM Tris, pH 8.5) Buffer.

B3. Linearization of desired P(acman) construct:

-10 µl vector (miniprep).
-5 µl Buffer 4 (NEB).
-2 µl AscI (10U/µl, NEB).
-2 µl PacI (10U/µl, NEB).
-31 µl MilliQ water.
-Incubate at 37°C for 120' or 180' if no dephosphorylation is performed.

Optional:
-Add 0.2 µl CIAP (Invitrogen, 10U/µl).
-Incubate at 37°C for 60'.

Dephosphorylation of vector is okay since digestion of homology arms generates phosphorylated ends, but the process is not really necessary. I omitted dephosporylation during the final plasmid preparation times.

B4. Purification of cut P(acman):

-Take 50 µl digestion.
-Add 5x Buffer PB (QIAGEN kit).
-Load everything on one single column (QIAGEN).
-Spin briefly (14.000 rpm, RT).
-Wash with 750 µl PE Buffer (QIAGEN).

-Spin briefly at 14.000 rpm, RT.
-Spin at 14.000 rpm, RT, 1'.
-Elute with 30 µl EB Buffer (10 mM Tris, pH 8.5).

-Check 2 µl on agarose gel.
-Store at –20°C.

B5. PCR for homology arms (TAQ ONLY SHOULD BE FINE):

Set up a gradient PCR (12 reactions, 44ºC to 66ºC) for each arm: Amplify preferably with Pfu (Stratagene), otherwise Taq polymerase (QIAGEN) or mix. PCR from target BAC verifies the presence of the homology arms in the target BAC. For BAC prep to obtain BAC DNA for PCR, see below.

B5.1 Pfu (Stratagene):

1 µl template (1/100 dilution of BAC prep)	13 µl
1 µl forward primer	13 µl
1 µl reverse primer	13 µl
2 µl 10x Buffer	26 µl
0.16 µl dNTPs	2.08 µl
0.4 µl Pfu DNA Polymerase	5.2 µl
14.44 µl HPLC water	187.72 µl
20 µl total	260 µl total

B5.2 Taq Polymerase (Qiagen):

1 µl template (1/100 dilution of BAC prep)	13 µl
1 µl forward primer	13 µl
1 µl reverse primer	13 µl
2 µl 10x Buffer	26 µl
0.16 µl dNTPs	2.08 µl
0.04 µl Taq DNA Polymerase	0.52 µl
14.8 µl HPLC water	192.4 µl
20 µl total	260 µl total

B5.3 Taq/Pfu Polymerase (Qiagen/Stratagene):

1 µl template (1/100 dilution of BAC)	13 µl
1 µl forward primer	13 µl
1 µl reverse primer	13 µl
2 µl 10x Buffer	26 µl
0.16 µl dNTPs	2.08 µl
0.1 µl Taq DNA Polymerase	1.3 µl
0.1 µl Pfu DNA polymerase	1.3 µl
14.64 µl HPLC water	190.32 µl
20 µl total	260 µl total

B6. Gel extraction of obtained PCR products (QIAGEN kit protocol):

-Slice complete gradient out of the gel.
-Add 3x Buffer QG (QIAGEN kit).
-Load everything on one single column (QIAGEN).
-This requires multiple brief spins (14.000 rpm, RT).
-Wash with 750 µl PE Buffer (QIAGEN).
-Spin briefly at 14.000 rpm, RT.
-Spin at 14.000 rpm, RT, 1'.
-Elute with 50 µl EB Buffer (10 mM Tris, pH 8.5).

-Check 2 µl on agarose gel.

B7. RE digestion of homology arms (LA/RA) (WHEN HYBRID PCR, DO ASCI/PACI):

B7.1 LA arm digestion:

25 µl gel purified PCR product.
5 µl Buffer 4 (NEB).

2 µl AscI (10U/µl, NEB).
1.5 µl BamHI (20U/µl, NEB).
0.5 µl BSA (NEB).
16 µl HPLC water.
50 µl total.
Put at 37°C for 180' (in parallel with vector cut and optional dephosphorylation).

7.2 RA arm digestion:

25 µl gel purified PCR product.
5 µl Buffer 1 (NEB).
1.5 µl BamHI (20U/µl, NEB).
2 µl PacI (10U/µl, NEB).
0.5 µl BSA (NEB).
16 µl HPLC water.
50 µl total.
Put at 37°C for 180' (in parallel with vector cut and optional dephosphorylation).

B8. PCR purification of digested PCR products:

-Take entire digestion: 50 µl.
-Add 5x Buffer PB (QIAGEN kit).
-Load everything on one single column (QIAGEN).
-Spin briefly (14.000 rpm, RT).
-Wash with 750 µl PE Buffer (QIAGEN).
-Spin briefly at 14.000 rpm, RT.
-Spin at 14.000 rpm, RT, 1'.
-Elute with 30 µl EB Buffer (10 mM Tris, pH 8.5).
-Check 2 µl on agarose gel.

B9. Ligation into desired P(acman)-vector:

-1 µl linearized vector (desired P(acman) vector, AscI/PacI cut)
-3.5 µl LA (AscI/BamHI cut)
-3.5 µl RA (BamHI,/PacI cut)
-1 µl 10x T4 DNA Ligase Buffer (NEB)
-1 µl T4 DNA Ligase (400U/µl, NEB)
-Ligate ON at 16°C.

B10. Precipitation of ligation (OPTIONAL: if the amount of colonies is too little):

-10 µl ligation reaction.
-Add 1 µl 3M NaOAc pH7.0.
-Add 25 µl 100% EtOH (-20°C).
-Put at least for 30' at –80°C.
-Spin for 20', 14.000 rpm at 4°C.
-Remove SN.
-Wash pellet with 100 µl 70% EtOH
-Spin for 5', 14.000 rpm at RT.
-Remove SN.
-Air dry for 5'.
-Redissolve in 2 µl MilliQ Buffer (10 mM Tris pH 8.0).

B11. Tranformation into EPI300 electrocompetent cells:

-Transform 1 µl of ligation directly or precipitated ligation mixture (2 µl) in 50 µl EPI300 electrocompetent cells (commercially obtained or **SELF-MADE**).
-Transform in 1 mm electroporation cuvette, 1.8 kV, 200 Ohm, 25 µFD.
-Add 450 µl SOC medium.
-Let recover at 37°C for 60'.
-Spread 50 µl and rest on LB-plates containing appropriate antibiotics (A100).

B12. Screening of EPI300 colonies by colony PCR:

-Screen obtained single colonies by colony PCR using 'goi-LA-Seq-F' and 'goi-RA-Seq-R' primers.
-Touch colony with sterile tip, streak in single PCR well and streak as a backup on LB-plate containing appropriate medium (A100).
-Perform PCR reaction using master-mix containing the following components:

1 µl forward primer
1 µl reverse primer
2 µl 10x Buffer
0.16 µl dNTPs
0.04 µl Taq DNA Polymerase
15.8 µl HPLC water
20 µl total

-Only correctly ligated constructs (three-way ligation) will turn positive and therefore show a band on the gel.

B13. Induction of PCR positive EPI300 colonies:

-Take PCR positive colonies.
-Grow up ON at 37°C in 1.5 ml LB medium containing appropriate antibiotics (A100) in Falcon50.
-Take 0.5 ml out and set aside for glycerol stock.
-Add 9 ml of LB containing 2 to 10 µl CopyControl Solution (1000x, Epicentre) and appropriate antibiotics (A100) to the remaining 1 ml.
-Let grow at 37°C for 5 hrs.

B14. Miniprep and sequencing of PCR positive EPI300 colonies:

-Do regular QIAGEN miniprep.
-Add 250 µl P1.
-Add 250 µl P2.
-Incubate at RT for 5'.
-Add 350 µl N3.
-Spin at 14.000 rpm, 10', RT
-Load SN on column (QIAGEN)
-Spin briefly at 14.000 rpm, RT.
-Wash with 750 µl PE Buffer.
-Spin briefly at 14.000 rpm, RT.
-Spin at 14.000 rpm, RT, 1'.
-Elute with 50 µl EB (10 mM Tris, pH 8.5) Buffer.
-RE digestion (Release of insert, two arms (1 kb total))

1 µl plasmid
1 µl AscI
1 µl PacI
2 µl Buffer 4
15 µl HPLC water
20 µl total

-Sequence with T7 and T3 primers.

B15. Linearization of correct P(acman) plasmid containing both homology arms:

-Linearize 1000 ng plasmid with BamHI at 37°C for 120'.

-1000 ng plasmid.
-Add HPLC water up to 10 µl.
-6 µl Buffer BamHI.
-2 µl BamHI (20U/µl).
-51.4 µl HPLC water.
-0.6 µl BSA.

-Do a PCR purification on the entire aliquot.

-Add 300 µl PB Buffer.
-Load on column.
-Spin at 14.000 rpm for 30''.
-Wash with 750 µl PE Buffer.
-Spin at 14.000 rpm for 30''.
-Remove flow-through.
-Spin at 14.000 rpm for 60''.
-Elute with 50 µl EB buffer.
-Divide in two parts.
-Keep one aliquot and freeze at –20°C.
-Load other one completely on gel (Add 5 µl 50% glycerol to avoid float-out from gel well during loading and 5 µl loading dye)

B16. Induction of recombination functions:

A. **Bring BAC (pBeloBAC11 or pBAC3.6 derived) into recombineering bacteria (DY380).**
B. **Bring recombination functions into BAC containing cell (mini-lambda).**
C. **Fingerprint and colony PCR confirmation of original BAC DNA from DH10B and BAC DNA in recombineering background DY380 or DH10B/mini-lambda.**
D. **Induction/Non-induction of recombination functions in DY380 or mini-lambda containing DH10B cells.**
E. **Transformation of induced/uninduced bacteria (DY380 or mini-lambda containing DH10B cells).**

B16.A Bring BAC (pBeloBAC11 or pBAC3.6 derived) into recombineering bacteria

B16.A1 Isolation of BAC's from host strain (DH10B):

-Grow up a single colony (PCR confirmed) in 5 ml **(9 ml)** of culture ON at 37°C (Cl12.5).
-Spin down, 3.800 rpm, 10', Eppendorf, 4°C.
-Resuspend in 250 µl **(320)** P1 (+RNase).
-Add 250 µl **(320)** P2.
-Let stand for 5'.
-Add 350 µl **(450)** N3
-Spin at 14000 rpm, 4'.
-Take SN.
-Spin at 14000 rpm, 4'.
-Take SN.
-Add 750 µl isopropanol.
-Put at RT for 10'.
-Spin at 14000 rpm, 10'.
-Wash with 1 ml 70% EtOH.
-**Air dry** for 5' (do not use speedvac).
-Add 50 µl EB.
-Let rehydrate for a few minutes.
-Short spin to collect. Never freeze. Transform freshly.

B16.A2 Transformation of isolated BAC from DH10B into DY380:

-Grow two colonies ON at **30°C** (T10).
-Add 300 µl of ON culture to 14.7 ml LB (T10).
-Grow up at **30°C** for 4 hrs.
-Put on ice for 5'.
-Spin at 3.220 rpm, Eppendorf, 4°C, 10'.
-Add 1 ml of autoclaved MilliQ water.
-Resuspend by tapping.
-Add additional 24 ml of autoclaved MilliQ water.
-Spin at 3.220 rpm, Eppendorf, 4°C, 10'.

-Add 1 ml of autoclaved MilliQ water.
-Resuspend by tapping.
-Add additional 24 ml of autoclaved MilliQ water.
-Spin at 3.220 rpm, Eppendorf, 4°C, 10'.
-Add 1 ml of 10% glycerol.
-Resuspend by tapping.
-Put slurry in Eppendorf tube.
-Spin at 14.000 rpm, 30'', 4°C.
-Remove most of 10% glycerol up to 120 µl.
-Divide in 2 aliquots of 60 µl cells for two transformations.
-Transform 1 µl previously isolated BAC DNA (**Freshly prepared**).
-Add 450 µl LB (**Preferentially SOC**).
-Shake at **30°C** 1.5 to 2 hrs.
-Plate 50 µl and 450 µl out on plates (Cl12.5/T10)
-Let grow for 20 to 24 hrs at **30°C**. Should give multiple colonies.

B16.B Bring recombination functions into BAC containing cell

B16.B1 Transformation of mini-lambda in DH10B BAC containing cells:

-Grow two colonies ON (PCR confirmed) at **37°C** (Cl12.5).
-Add 300 µl of ON culture to 14.7 ml LB (Cl12.5).
-Grow up at **37°C** for 3 hrs.
-Put on ice for 5'.
-Spin at 3220 rpm, Eppendorf, 4°C, 10'.
-Add 1 ml of autoclaved MilliQ water.
-Resuspend by tapping.
-Add additional 24 ml of autoclaved MilliQ water.
-Spin at 3220 rpm, Eppendorf, 4°C, 10'.
-Add 1 ml of autoclaved MilliQ water.
-Resuspend by tapping.
-Add additional 24 ml of autoclaved MilliQ water.
-Spin at 3220 rpm, Eppendorf, 4°C, 10'.
-Add 1 ml of 10% glycerol.
-Resuspend by tapping.
-Put slurry in Eppendorf tube.
-Spin at 14000 rpm, 30'', 4°C.
-Remove most of 10% glycerol up to 120 µl.
-Divide in 2 aliquots of 60 µl cells for two transformations.
-Transform 2 µl mini-lambda (Tet).
-Add 450 µl LB **(Preferentially SOC)**.
-Shake at **30°C** 1.5 to 2 hrs.
-Plate 50 µl and 450 µl out on plates (Cl12.5/T10)
-Let grow for 20 to 24 hrs at **30°C**. Should give multiple colonies.

B16.C Fingerprint and colony PCR confirmation of original BAC DNA from DH10B and BAC DNA in recombineering background DY380 or DH10B/mini-lambda.:

Fingerprint:
17.5 µl DNA
0.5 µl EcoRI (NEB)
2 µl Buffer EcoRI (NEB)
Compare to DNA isolated from host strain (DH10B)

Colony-PCR using primers of both homology arms.

B16.D Induction/Non-induction of recombination functions in DY380 or mini-lambda containing DH10B cells:

-Grow single colony ON (**30°C**).
-Add 300 µl of ON culture to 14.7 ml LB (Cl12.5/T10).
-Do this in duplicate: induced versus uninduced.
-Grow up at **30°C** for 3 hrs (usually enough. Optimal OD_{600} is between 0.4 and 0.6. Tend to aim for 0.5).
-Induce at **42°C** (in a shaking waterbath) for 15'. Keep uninduced at **30°C** for 15'.
-Shake in ice-water slurry for 5'.
-Spin at 3220 rpm, Eppendorf, 4°C, 10'.
-Add 1 ml of autoclaved MilliQ water (4°C).
-Resuspend by tapping.
-Add additional 24 ml of autoclaved MilliQ water (4°C).
-Spin at 3220 rpm, Eppendorf, 4°C, 10'.
-Add 1 ml of autoclaved MilliQ water (4°C).
-Resuspend by tapping.
-Add additional 24 ml of autoclaved MilliQ water (4°C).
-Spin at 3220 rpm, Eppendorf, 4°C, 10'.
-Add 1 ml of 10% glycerol (4°C).
-Resuspend by tapping.
-Put slurry in Eppendorf tube.
-Spin at 14000 rpm, 30'', 4°C.
-Remove most of 10% glycerol up to 120 µl.
-Divide in 2 aliquots of 60 µl cells for two transformations.

B16.E Transformation of induced/uninduced bacteria (DY380 or mini-lambda containing DH10B cells):

-Transform 2 µl of linearized P(acman) in 60 µl induced/uninduced bacteria.
-Transform in 1 mm electroporation cuvette, 1.8 kV, 200 Ohm, 25 µFD.
-Add 450 µl LB medium (**Preferentially SOC**).
-Let recover at **30°C** for 90 to 120'.
-Spread all on LB-plates containing appropriate antibiotics (A100).
-Let grow ON at **30°C** (can take 48 hours. I usually see small/medium colonies after 24 hrs most of the time).

B17. PCR screening of recombinants:

-Screen obtained single colonies by colony PCR using **MCS-F/'goi-5'-check-R'** for 5' gap-repair and **'goi-3'-check-F'/MCS-R** for 3' gap-repair.
-Touch colony with sterile tip, streak in single PCR well (2 times) and streak as a back-up on LB plate containing antibiotic of the gap-repaired plasmid (A100).
-Perform PCR reaction using master-mix containing the following components:

1 µl forward primer
1 µl reverse primer
2 µl 10x Buffer
0.16 µl dNTP's
0.04 µl Taq DNA Polymerase
15.8 µl HPLC water
20 µl total

-Only correctly recombined plasmids should turn positive for both PCRs.

B18. Grow up PCR confirmed recombinants and isolate DNA from recombinogenic strain:

-Grow up a single colony in 5 ml of culture ON (30°C).
-Spin down, 3800 rpm, 10', Eppendorf, 4°C.
-Resuspend in 250 µl P1 (+RNase)
-Add 250 µl P2
-Let stand for 5'.

-Add 350 µl N3.
-Spin at 14.000 rpm, 4'.
-Take SN
-Spin at 14.000 rpm, 4'.
-Take SN.
-Add 750 µl isopropanol.
-Put at RT for 10'.
-Spin at 14.000 rpm, 10'.
-Wash with 1 ml 70% EtOH.
-Air dry for 5' (No Speedvac).
-Add 50 µl TE/EB.
-Rehydrate for a few minutes.
-Short spin to collect. **Transform freshly. Never freeze.**

B19. Transform obtained plasmid in EPI300 cells:

-Transform 0.5 µl of isolated DNA in 50 µl EPI300 electrocompetent cells (commercially obtained or **SELF-MADE**).
-Transform in 1 mm electroporation cuvette, 1.75 kV, 200 Ohm, 25 µFD.
-Add 450 µl LB medium (Preferentially SOC).
-Let recover at 37°C for 60'.
-Spread 20-30 µl on LB-plates containing appropriate antibiotics (Usually gives a lot of colonies, depending on the size of the plasmid).

B20. PCR screening of bacteria containing only correct recombined plasmid:

-Screen obtained single colonies by colony PCR using **MCS-F/'goi-5'-check-R'** for 5' gap-repair and **'goi-3'-check-F'/MCS-R** for 3' gap-repair.
-Touch colony with sterile tip, streak in single PCR well (2 times) and streak as a back-up on LB-plate (A100) and test for absence of Chl (C12.5).
-Perform PCR reaction using master-mix containing the following components:

1 µl forward primer
1 µl reverse primer
2 µl 10x Buffer
0.16 µl dNTP's
0.04 µl Taq DNA Polymerase
15.8 µl HPLC water
20 µl total

-Only correctly recombined plasmids should turn positive for both PCRs and are Amp positive and Chl negative.

B21. Induction of plasmid:

-Take PCR positive colonies.
-Grow up ON at 37°C in 1.5 ml LB medium containing appropriate antibiotics (A100).
-Take 0.5 ml and set aside for glycerol stock.
-Add 9 ml of LB containing 2 to 10 µl CopyControl Solution (Epicentre) and appropriate antibiotics (A100) to the remaining part of the culture).
-Let grow at 37°C for 5 hrs.

B22. Isolation of plasmid (this is for plasmid verification by fingerprinting and sequencing, not microinjection):

B22.1 Plasmid smaller than 20 kb: Regular Qiagen miniprep:

-Add 250 µl P1.
-Add 250 µl P2.
-Incubate at RT for 5'.
-Add 350 µl N3.
-Spin at 14.000 rpm, 10', RT

-Load SN on column (QIAGEN)
-Spin briefly at 14.000 rpm, RT.
-Wash with 750 µl PE Buffer.
-Spin briefly at 14.000 rpm, RT.
-Spin at 14.000 rpm, RT, 1'.
-Elute with 50 µl EB (10 mM Tris, pH 8.5) Buffer.

B22.2 Plasmid larger than 20 kb up to 50 kb (has worked up to 90 kb, but increased size results in decreased yield): Adapted miniprep protocol:

-Add 450 µl P1.
-Add 450 µl P2.
-Incubate at RT for 5'.
-Add 630 µl N3.
-Spin at 14000 rpm, 10', RT
-Load SN on column (QIAGEN), 2x.
-Spin briefly at 14.000 rpm, RT.
-Wash with 0.5 ml PB Buffer.
-Spin at 14.000 rpm, RT, 30''.
-Wash with 750 µl PE Buffer.
-Spin at 14.000 rpm, RT, RT, 30''.
-Spin at 14.000 rpm, RT, 1'.
-Elute with 50 µl EB (10 mM Tris, pH 8.5) Buffer preheated at 70°C.

B22.3 Plasmid larger than 50 kb: PCI protocol:

-315 µl P1.
-315 µl P2.
-441 µl N3.
-Centrifuge for 4' at full force.
-Take SN.
-Centrifuge for 4' at full force.
-Take SN.
-Prespin 2 PLG (Phase Lock Gel) heavy 2 ml columns at 4500 rpm for 2'.
-Add SN to 700 µl PCI.
-Invert 20x.
-Centrifuge for 5' at full force.
-Add aqueous phase to 700 µl chloroform.
-Invert 20x.
-Centrifuge for 5' at full force.
-Take aqueous phase.
-Add 750 µl isopropanol.
-Put 10' at RT.
-Centrifuge for 10' at full force.
-Wash with 1 ml 70% EtOH.
-Centrifuge for 5' at full force.
-Air dry for 5' on high.
-Add 50 µl EB buffer and incubate 120' at 65°C.

B23. Verification of correctly recombined plasmid:

B23.1 Restriction enzyme fingerprint:

-Do AscI/PacI digestion. This releases the insert.
-Do AscI/EcoRI digestion. This gives fingerprint.

B23.2 Sequencing:

-Sequence plasmid with goi-LA-Seq-F and goi-RA-Seq-R. This will give sequence from inside the homology arm into the gap-repaired insert.