## *GC-Rich Genomes* Cloning Techniques –p15Tv-L

### 1. PCR Amplification

| | /Rxn | |
|---|---|---|
| $H_2O$ | 34.1µl | *Make a master mix, aliquot 48µl/rxn |
| 10x Pfx buffer | 5µl | and add: 1.0µl forward primer (50µM) |
| 50 mM $MgSO_4$ | 1µl | 1.0µl reverse primer (50µM) |
| 10 mM dNTP's | 1.5µl | |
| 10x Enhancer | 7.5µl | |
| genomic dna (100ng/µl) | 0.4µl | |
| Pfx (2.5U/µl) | 0.5µl | |
| | 50µl | |

PCR Program:

1. 95° 2:30 min.
2. 95° 15 sec.
3. 53° 30 sec.
4. 68° 1 min./kb
5. Go To 2 Rep. 29x
6. 72° 10 min
7. Hold 4°

### 2. Run Large Gel – Verification of PCR product size

1% gel

| | | | |
|---|---|---|---|
| Large: | 3 grams | Small: 0.5 grams | Agarose |
| | 300 ml | 50 ml | 1x TAE |

i. Heat in microwave 4½~5 min.
ii. Let cool in cold room ~20 min.
iii. Load 5 µl of PCR product with 2µl of 6x SYBR green loading dye
iv. Run gel @220-240 ~30 min. (large gel), @120V ~20min. (small gel)
v. View gel using gel doc

**3. PCR Clean-up**

1. Transfer 45µl PCR product to round bottom deep well
2. Add 3:1 Buffer PM (135µl), mix on vortexer
3. Transfer to Qiagen Qiaquick 96 filter plate
4. Turn on vacuum for 5-7 min.
5. Add 900µl Buffer PE, and vacuum for 5 min.
6. Repeat above
7. Dispose of ethanol in waste tray, and vacuum 20~30 min.
8. Make sure all residual ethanol is gone by tapping on paper towel
9. Elute with 50µl of EB buffer

Or

1. Transfer 45µl PCR product to 1.5 ml microcentrifuge tube
2. Add 5:1 Buffer PB (225 µl)-refer to manual, mix by pipetting
3. Transfer to QIAquick Spin Columns (pink) and centrifuge @ 14,000 rpm for 1min
4. Add 750 µl Buffer PE, centrifuge @ 14,000 rpm for 1min
5. Discard ethanol and centifuge again @ 14,000 rpm for 1min
6. Transfer column to a clean 1.5ml microcentrifuge tube
7. Let sit at room temperature for ~ 30 min.
8. Elute with 50 µl of EB buffer

**4. Infusion Reaction – Annealing vector and insert**

1. Aliquot 4.0 ul of PCR target into pcr tube/plate.
2. Dissolve BD-Infusion pellet with 8.5ul of prepared vector. Use 2ul of the infusion/vector mix and add to each insert (Therefore each infusion pellet can be used for 4 reactions).
3. Incubate for 30 min at 28ºC, then place reactions on ice or freeze.

**5. Transformation**

1. Place PCR plate containing ligation on ice ~10 min.
2. Add 80µl of competent DH5α to each ligation
3. Leave on ice for 10 min.
4. Heat shock @ 42° for 45sec.
5. Place on ice for 2 min.
6. Recover in 900µl of SOC for 1 hr. @ 37° with shaking
7. Spin down @ 3000rpm for 10 min.
8. Discard supernatant leaving 55 µl to plate on LB+carb(100omg/ml)+sucrose(5%) plates
9. Incubate plates O/N @ 37°

**6. Colony Screen**

| | /Rxn | |
|---|---|---|
| $H_2O$ | 16.2µl | *Make a master mix, aliquot 28µl/rxn |
| Taq buffer | 3µl | and add colony to each tube |
| 25 mM $MgCl_2$ | 2.4µl | |
| 2 mM dNTP's | 3µl | |
| 10x Enhancer | 4.5µl | |
| T7 promoter (~600µg/ml) | 0.3µl | |
| T7 terminator (~600µg/ml) | 0.3µl | |
| Taq (5U/µl) | 0.3µl | |
| | 30µl | |

PCR Program:

1. 94° 2 min.
2. 94° 30 sec.
3. 50° 30 sec.
4. 68° 1 min./kb
5. Go To 2 Rep. 29x
6. 72° 10 min
7. Hold 4°

**7. Run Large Gel – Verification of positive clones**

1% gel

3 grams Agarose
300 ml 1x TAE

i. Heat in microwave 4½~5 min.
ii. Let cool in cold room ~20 min.
iii. Load 5 µl of PCR product with 2µl of 6x SYBR green loading dye
iv. Run gel @220-240 ~30 min.
v. View gel using gel doc

**8. Grow**

*Pick positive colony for each clone, and add to 1ml of LB+amp in deep well
*Shake O/N @37°

**9. Plasmid Prep of Positive Clones**

1. Spin O/N grown plate for 10 min. @ 3000rpm
2. Discard supernatant
3. Add 250µl P1 buffer and vortex to resuspend pellet
4. Add 250µl P2 buffer (lysis buffer) and shake slowly, no more than 5 min.
5. Add 350µl N3 buffer (neutralization buffer) and shake slowly
6. Transfer (850 µl) to Qiagen Turbofilter plate (white), with Qiaprep plate (blue) beneath
7. Vacuum for 3-5 min.
8. Discard TurboFilter, and place Qiaprep plate on top
9. Vacuum for ~10 min.
10. Add 900µl PE buffer, and vacuum 3 min.
11. Repeat above step
12. Elute with 100µl buffer EB

**Preparation of p15TV-L vector (one-96 plate)**

1. Grow 3 x 4ml overnight cultures of p15Tv-L from single colony.
2. Miniprep using Qiagen spin columns, using 4 ml of culture per column. Elute with 50ul of EB per column.
3. Digestion:

| | | |
|---|---|---|
| p15Tv-L | 120 ul | |
| 10xNEB#2 | 60 ul | |
| BseRI | 15 ul | |
| $H_20$ | 405 ul | (1.5 hrs @ 37°C) |

Add

| | | |
|---|---|---|
| 10xNEB#2 | 10 ul | |
| BseRI | 5 ul | |
| $H_20$ | 85 ul | (1.5 hrs @ 37°C) |
| | 700 ul | |

4. Transfer to 15 ml conical falcon tube, add 3.5ml of PB buffer
5. Purify by transfering 700ul each to 6 Qiagen spin columns, elute with 50ul EB per column.

**2mM dNTPs**

900µl $H_2O$
25µl ea. dNTP (100mM ea.)
1000µl

**10mM dNTPs**

150µl $H_2O$
25µl ea. dNTP (100mM ea.)
250µl

**6x SYBR Green Dye**

0.125g bromophenol blue
0.125g xylene cyanol FF
15 ml glycerol
30µl 10,000x Vistragreen
bring up to 50ml with $dH_2O$

**50x TAE**

242g Tris base
57.1ml glacial acetic acid
18.6g EDTA
bring up to 1L with $dH_2O$

**1x TAE**

20ml of 50x TAE
bring up to 1L with $dH_2O$

**LB broth (Difco)**

15.5g LB powder
bring up to 1L with $dH_2O$
(autoclave)

**LB Agar (BioShop)**

40g LB powder
bring up to 1L with $dH_2O$
(autoclave)

**SOC (Difco)**

28g SOB powder
bring up to 1L with $dH_2O$
(autoclave)
*once cool turn SOB into SOC
by adding 20ml of 20% glucose

**20% Glucose**

20g glucose in 100ml $dH_2O$

**Stock Ampicillin** (100µg/µl)

1g amp in 10ml $dH_2O$
*filter sterilize -0.2
*aliquot and store at -20°

# Competent Cells

1. Inoculate 2ml LB from a single colony on a plate, and grow O/N @ 37° with shaking
2. Take 2ml of O/N culture and transfer to 400ml LB
3. Shake @ 37° until $OD_{600}$ ~0.4

*All steps are done in cold room and aseptically

4. Collect 50 ml of culture in cold falcon tubes, and keep on ice for 10 min.
5. Spin @ 3000rpm for 10 min. @ 4°. Pour off supernatant.
6. Gently resuspend cells with 20ml/tube RF1 buffer
7. Keep on ice for 60 min.
8. Spin @ 3000rpm for 10 min @ 4°. Pour off supernatant.
9. Gently resuspend pellet in 4ml/tube RF2 buffer
10. Aliquot out 500µl of resuspended cells into cold 1.5ml microcentrifuge tubes, place in floater
11. Place floater in liquid nitrogen for 1 min., remove tubes and store @ -80°

## RF1 Buffer

| | Per 100ml | [Final] |
|---|---|---|
| RbCl | 1.2g | 100mM |
| $MnCl_2 \cdot 4H_2O$ | 1.0g | 50mM |
| KOAc | 3ml of 1M stock @ pH7.5 | 30mM |
| $CaCl_2 \cdot 2H_2O$ | 0.15g | 10mM |
| Glycerol | 15 ml | 15% |

*Adjust pH to 5.8 with 0.2M acetic acid
*Filter sterilize

## RF2 Buffer

| | Per 100ml | [Final] |
|---|---|---|
| MOPS | 2ml of 0.5M stock @ pH6.8 | 10mM |
| RbCl | 0.12g | 10mM |
| $CaCl_2 \cdot 2H_2O$ | 1.1g | 75mM |
| Glycerol | 15 ml | 15% |

*Adjust pH to 6.8 with NaOH
*Filter sterilize