While Matt’s larking off in Denmark, I will try to carry his cloning forward.

5.15.15 Onward and Upward – Cut and SAP

He has amplified and purified the Methanosphaera stadmanae artificial mta operon. The product is 5,976bp and his concentrations are 877ng/μL (tube 1 of 2) and 887ng/μL (tube 2 of 2).

I’ll supply the vector, pAW42C (prep 3 of 3 from 4.28.15), which is 4,934bp and has a concentration of 1,247ng/μL.

**Molar-ends calculations (#’s in red are specific to the DNA in question)**

μg DNA x 1pmol x 106pg x 1 x 2 x 1 kb

660pg μg N 1000bp

* N is the number of nucleotides (**in kb**)
* 660 pg/pmol is the average molecular weight of a single nucleotide pair
* 2 is the number of ends in a linear DNA molecule
* kb/1000bp is a conversion factor for kilobases to base pairs

**Insert Ends Calculation**

877μg/mL x 1/660 x 106 x 1/5.98 x 2 x 1/1000

= **444 pmoles of ends / 1μL of purified DNA** [this calculation was for prep 1 of 2, shown above]

**Vector Ends Calculation**

**1,247μg/mL x 1/660 x** 106 x 1/4.93 x 2 x 1/1000

= **766 pmoles of ends / 1μL of purified DNA**

Cut with *Nsi*I and *Bgl*II:

DNA Species

Reagent Vector Insert

water 36.5 34.5

\*of a 1:10 dilution of the vector prep.

10X Buffer 3.1 4.5 4.5

DNA prep 1.0\* 3.0

*Nsi*I 1.5 1.5

*Bgl*II 1.5 1.5

Total volume 45.0μL 45.0μL

total pmoles ends 76.6 1,332

pmoles ends/μL 1.70 29.6

Incubated 1 hour at 37ºC, then accidentally added 56μL of sterile water to the vector digest rather than transferring half the vector digest to a fresh tube and THEN adding the water. So the new numbers for the digests are:

Reagent Vector Insert

Total volume 101.0μL 45.0μL

total pmoles ends 76.6 1,332

pmoles ends/μL 0.758 29.6

With a few recalculations, I carried on as follows: I transferred 23μL of the vector reaction to a fresh tube, then add the following reagents to the transferred half of the vector reaction:

Reagent Vector Insert

*Nsi*I/*Bgl*II rxn 23.0

water 103.5 --.--

10X Buffer 3.1 26.5 --.--

rSAP 17.0 --.--

New Total volume 170.0μL 45.0μL

total pmoles ends 17.4 1,332

**pmoles ends/μL 0.102 29.6**

Incubated a further 30’ at 37ºC.

Inactivated enzymes at 80ºC for 20’. Stored at -20ºC overnight.

5.21.15 Ligation and Transformation

Vector : Insert ratio 🠂 1:1 1:29

Water 9.0 9.0

10X Fast-Link Buffer 1.5 1.5

10mM ATP 1.5 1.5

vector DNA 1.0 = 0.102pmole ends 1.0 = 0.102 pmole ends

insert DNA 1.0\* = 0.102pmole ends 1.0\*\* = 2.96 pmole ends

Fast-Link Ligase 1.0 1.0

Total volume (μL) 15.0μL 15.0μL

\*of a 1:290 dilution \*\*of a 1:10 dilution

Incubated 20’ on bench then inactivated ligase 15’ at 75ºC.

Transformed 2μL of each ligation reaction into 70μL of XL10 Gold competent cells. Plated 50μL of each recovery culture on LAamp plates.

5.22.15 Screening PC reactions

Only 5 colonies, all about the same size, grew on the 1:1 plating, while the 1:20 plate had 11 colonies, most were smallish. there were two to three colonies in the larger size range and these were about the same size as those on the 1:1 plate. I concentrated and plated the remainder of each recovery culture and will screen those on Sunday, if need be. Judging from past experience with these operon insertions, it will be necessary.

In the meantime, I ran pc reactions to screen the 16 candidates I had in hand, plus a vector control:

Volume (µL)

Reagent 1 rxn 19 rxns

water 31.5 598.5

5X iProof HF Buffer 10.0 190.0

10mM dNTP’s 1.0 19.0

fwd primer\* 1.0 19.0

rev primer\*\* 1.0 19.0

template\*\*\* 5.0 --.---

iProof polymerase 0.5 9.5

Total volume 50.0

\* Forward primer = EGP55.

\*\* Reverse primer = EGP56.

\*\*\* Boil-preps of candidate colonies; the no-insert control was pJAR50C

Thermalcycler program:

Cycle Temperature Time # Cycles

initial denaturation 98ºC 1’ 1

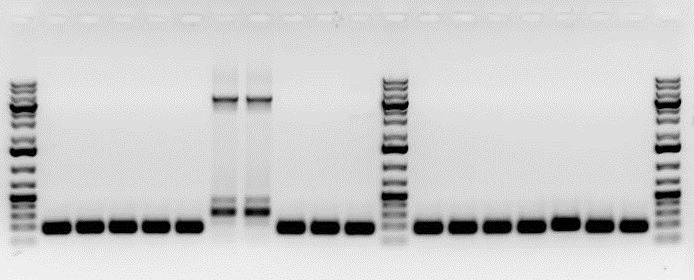
denaturation 98ºC 10”

annealing **53ºC** 10” 35

extension 72ºC 2’

final extension 72ºC 1’ 1

1. GeneRuler 1kb Plus
2. candidate #1



Ladder:

20,000

10,000

7,000

**5,000**

4,000

3,000

2,000

**1,500**

1,000

700

**500**

400

300

200

75

1. #2
2. #3
3. #4
4. #5
5. **#21**
6. **#22**
7. #23
8. #24
9. pJAR50C control
10. GeneRuler 1kb Plus
11. #25
12. #26
13. #27
14. #28
15. #29
16. #30
17. #31
18. GeneRuler 1kb Plus

Streaked candidates 21 and 22 on LAamp for colony purification.

5.26.15 Beginning subclone into pJAR50CT

Will use the same stock of cut insert DNA as above and simply cut the new vector. The vector used here was purified on 5.24.15 (prep 1 of 3; isolate 2M), and this prep had a concentration of 1,380μg/mL. The vector is 5078bp.

**Vector Ends Calculation**

**1,380μg/mL x 1/660 x** 106 x 1/5.08 x 2 x 1/1000

= **823 pmoles of ends / 1μL of purified DNA**

Cut pJAR50CT vector with *Nsi*I and *Bgl*II:

Reagent Volume

water 40.0

\*of a 1:10 dilution of the vector prep.

10X Buffer 3.1 5.0

DNA prep 1.0\*

*Nsi*I 2.0

*Bgl*II 2.0

Total volume 50.0μL

total pmoles ends 82

pmoles ends/μL 1.6

Reagent Vector Insert ‡

‡ Same prep as that on pages 1 and 2 above. No further manipulations performed here.

*Nsi*I/*Bgl*II rxn 10.0

water 118.0 --.--

10X Buffer 3.1 16.0 --.--

rSAP 16.0 --.--

New Total volume 160.0μL 45.0μL

total pmoles ends 16 1,332

**pmoles ends/μL 0.1 29.6**

Incubated a further 30’ at 37ºC.

Inactivated enzymes at 80ºC for 20’. Stored at -20ºC overnight.

5.26.15 Ligation and Transformation

Vector : Insert ratio 🠂 1:29

Water 9.0

10X Fast-Link Buffer 1.5

10mM ATP 1.5

vector DNA 1.0 = 0.100pmole ends

insert DNA 1.0\* = 2.96pmole ends

Fast-Link Ligase 1.0

Total volume (μL) 15.0μL

\*of a 1:10 dilution

Incubated 20’ on bench then inactivated ligase 15’ at 75ºC.

Transformed 2μL of each ligation reaction into 70μL of XL10 Gold competent cells. Plated 50μL and, separately, the remainder of each recovery culture on LAamp plates.