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Primer Design Strategy

Design Methods

The two plasmids being used for developing this specific cyanobacterium are pAM4788 as the backbone vector and pCS-PET_VB190412 for the superPETase gene. For convenience's sake, they shall be referred to in this document as pAM and PET, respectively. In order to design these primers, two steps must be taken. The first step is to identify the regions of each plasmid necessary for PCR and Gibson cloning. The second step is to isolate the specific fragments of each plasmid that will be used in the PCR and cloning protocols.

Region Identification

In the PET plasmid, the region to be isolated is that of the designed superPETase gene (shown in yellow in Figure 1).

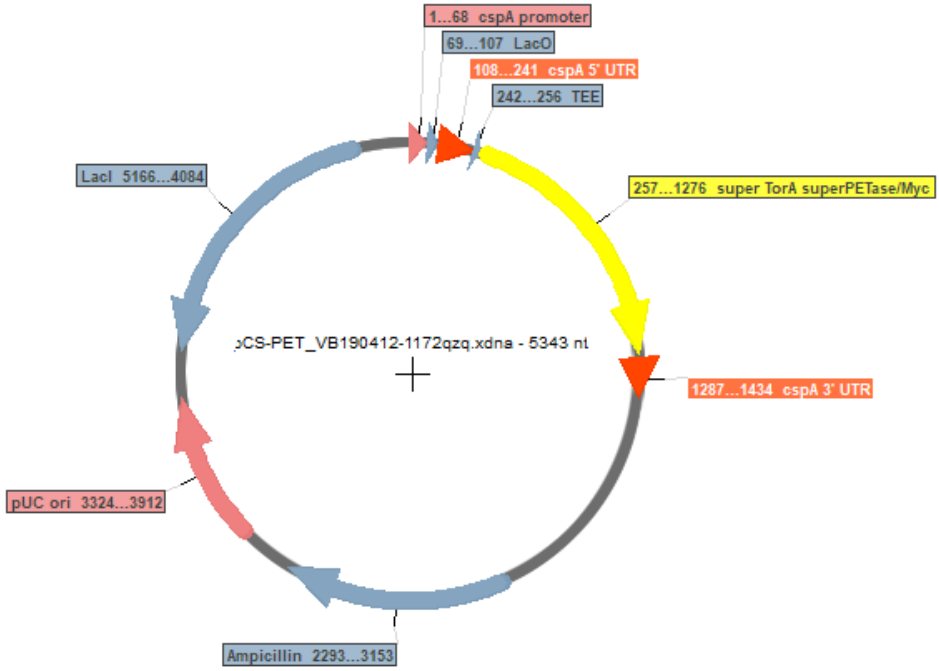

Figure 1. Graphic map of the pCS-PET_VB190412 plasmid identifying featured regions.

In the pAM plasmid, the region to be removed is the GFP gene (shown in green in Figure 2), leaving the rest of the plasmid as the backbone vector. The superPETase gene (yellow) is inserted where the GFP gene (green) was removed.

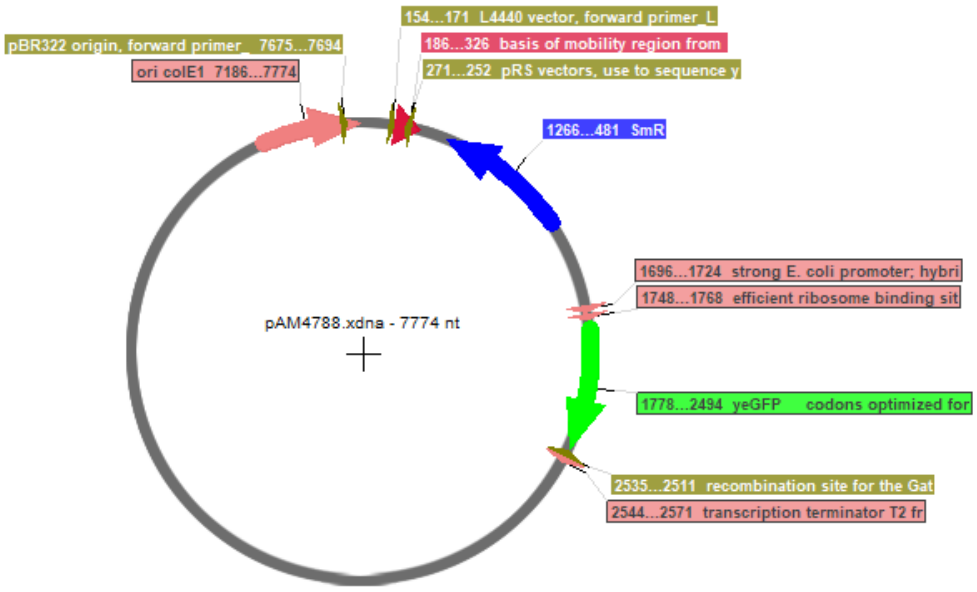

Figure 2. Graphic map of the pAM4788 plasmid identifying featured regions.

Fragment Isolation

From the entire plasmid sequence, the fragments of interest are those at the beginning and end of the identified regions. The entire sequences of both the PET plasmid and the pAM plasmid are available in the appendix. The first sequence is that of the PET plasmid with the superPETase gene text in maroon and highlighted in yellow. The second sequence is that of the pAM plasmid with the GFP gene text in green and highlighted in yellow.

PCR primer isolation

The basic requirements for a PCR primer sequence is that n ≥ 18 and that T_m ≥ 55°C, where n is the number of base pairs and T_m is the melting temperature. A forward and reverse primer must be made for each fragment that is desired to be replicated. For the superPETase gene, the primers would be composed of the sequences of base pairs at the beginning and ending of the gene. For the pAM plasmid backbone, the primers would be composed of the sequences of base pairs just before and just after the GFP gene.

<u>superPETase primers</u>

Forward primer

```
                atggcaaacaacgacctattcc    n = 22 Tm = 55.7°C
catgaatcacaaagtgatggcaaacaacgacctattccaagccagtcgccgccgttttctggcacagctgggggggctca  < 320
          250       260       270       280       290       300       310
```

PET_f: ATG GCA AAC AAC GAC CTA TTC C

Reverse primer

```
                    {aactcatctcagaagaggatctgtaa}  n = 26 Tm = 55°C
cggccaattgcagtgaacaaaaactcatctcagaagaggatctgtaatctagataggtaatctctgcttaaaagcacagaat < 1310
1230      1240      1250      1260      1270      1280      1290      1300
```

PET_r: TTA CAG ATC CTC TTC TGA GAT GAG TT

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pAM backbone primers

Original forward primer

```
                aagggtgggcgcgcccgac  n = 18 Tm = 67.1°C
aattgtacaaataaaagggtgggcgcgcccgacccagctttcttgtacaaactcggccctcaggagaaggccatcctgacg  < 2560
          2490      2500      2510      2520      2530      2540      2550
```

pAM_f: AAG GGT GGG CGC GCC GAC

However, after consulting with NM, the primer-dimer potential became apparent as shown below:

```
5' AAGGGTGGGCGCGCCGACCCA
       ||||   ::   ::::
3'     ACCCAGCCGCGCGGGTGGGAA
```

To resolve this issue, an additional 4 base pairs were added at the 3' end of the original primer

pAM_f: AAG GGT GGG CGC GCC GAC CCA G

pAM_f2

ok so we are finding some mispriming sites with pAM_f. all reactions with this primer give two bands, second product ~ 500 bp smaller.

We could possibly fix this by tweaking annealing temperature and other program features. But it is probably easiest/quickest to just fix primer.

One issue is that AM_f is in the MCS, so it is challenging to avoid primer dimer due to the large number of palindromes. Extending AM_f by 1 or 2 bp causes severe primer dime issue (1 bp: GC bad; 2 bp AGCT terrible!). So let's extend by 3 or 4… pick 4. This is pAM_f2

```
AAGGGTGGGCGCGCCGACCCAGCTTT   AM_f2
AAGGGTGGGCGCGCCGACCCAG   AM_f
     gggcgcgccgacccagctttcttgtacaaactcggccctcaggagaaggccatcctgacggatggccttttgaagctttctagaatcgggggccccggg  < 2600
          2510      2520      2530      2540      2550      2560      2570      2580      2590

     ggggattctagaccgagctgggcactgacagcctcagtgagcggctctcggaagcgctcgagcattggccacatgtgcttgggtgctgcgatggccagcg  < 2700
          2610      2620      2630      2640      2650      2660      2670      2680      2690

     ttccactatcgaagctgtaggcgcggcagcggttagccgcaaaccatcgcaagccttcgttctcgatcgcggcgatcgcagttgtccaaagctgatcgag  < 2800
          2710      2720      2730      2740      2750      2760      2770      2780      2790

     ttcggcttgtgtcatgctgcggtctccgtagatgtagtgctgacctgacctgacgcggtgtcacgaaaatgcgcagtgattggttttgatcttctattag  < 2900
          2810      2820      2830      2840      2850      2860      2870      2880      2890

   AAGGGTGGGCGCGCCGACCCAGCTTT   AM_f2
AAGGG---TGGGCGCGCCGACCCAG   AM_f mispriming site??          AAGGGTGGGCGCGCCGACCCAG
     ggatttcgtgacagctccccagtccctgactagatcgggcgttttcaagccaatgagcttcgatcgcctcaagcggcagttcagcaatcgcctcattcgg  < 3000
          2910      2920      2930      2940      2950      2960      2970      2980      2990
```

pAM_f2: AAGGGTGGGCGCGCCGACCCAGCTTT

Original reverse primer

```
          {ctttaagaaggagcccttcacc}  n = 22 Tm = 55.4°C
ccttgtttaactttaagaaggagcccttcaccatgtctaaaggtgaagaattattcactggtgttgtcccaattttggttgaattagatggtgat  < 1840
    1750      1760      1770      1780      1790      1800      1810      1820      1830
```

pAM_r: GGT GAA GGG CTC CTT CTT AAA G

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The primer-dimer potential for this sequence was very high, as shown below:

```
5’-GGTGAAGGGCTCCTTCTTAAAG-3’
          *** ***
3’-GAAATTCTTCCTCGGGAAGTGG-R’
```

This was resolved by adding an A at the 3’ end of the original primer before converting into the complement
pAM_r: GGT GAA GGG CTC CTT CTT AAA GT

<u>pAM Internal Primers</u>

Running PCR amplification for the pAM backbone consist of >7k base pairs. This is too large to be efficient. To compensate for this, we divided the pAM backbone into 2 separate fragments and created primers. The primers are as follows:

Note- naming convention: 35K refers to 3.5 kbp past pAM_f

Forward primer (pAM35k_f)

```
                                                    CGAATTGATCGGCAAGCCAG   pAM35k_f n = 20 Tm = 56.9°C
gctttgattgccgataactatagctggaatgaatacggcaggtggcaaggctgcctcgaattgatcggcaagccagaggcgcgatcgcccctggctgtgatgagacttaga
cgcc
        5950      5960      5970      5980      5990          6010      6020      6030      6040      6050
```

pAM35k_f: CGA ATT GAT CGG CAA GCC AG

Reverse primer (pAM35k_r)

```
                                                    {CGAATTGATCGGCAAGCCAG}  pAM35k_r n = 20 Tm = 56.9°C

gctttgattgccgataactatagctggaatgaatacggcaggtggcaaggctgcctcgaattgatcggcaagccagaggcgcgatcgcccctggctgtgatgagacttaga
cgcc
        5950      5960      5970      5980      5990          6010      6020      6030      6040      6050
```

{CGAATTGATCGGCAAGCCAG}
Complement: CTG GCT TGC CGA TCA ATT CG
pAM35k_r: CTG GCT TGC CGA TCA ATT CG

In addition to splitting the pAM backbone in half, it was also split into three parts, with the primers designed as shown below.

Forward primer (pAM27k_f)

```
                                GCAGGTACATGGGCCAGCAT      pAM27k_f n = 20 Tm = 60.5°C
gccacccagctctcgtagtcgtctgcgtagctggcaggtacatgggccagcatggtgcgggcgatcgcgacctcatcatcagaagctggagcactgacgatcg
g
5160      5170      5180      5190      5200      5210      5220      5230      5240      5250
```

pAM27k_f: GCA GGT ACA TGG GCC AGC AT

Reverse primer (pAM27k_r)

```
                                {GCAGGTACATGGGCCAGCAT}      pAM27k_r n = 20 Tm = 60.5°C
gccacccagctctcgtagtcgtctgcgtagctggcaggtacatgggccagcatggtgcgggcgatcgcgacctcatcatcagaagctggagcactgacgatcg
g
5160      5170      5180      5190      5200      5210      5220      5230      5240      5250
```

pAM27k_r: ATG CTG GCC CAT GTA CCT GC

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	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer 23K_f	GCTGATATTCCCGCTGCTCT	Plus	20	2450	2469	59.97	55.00	4.00	0.00
Reverse primer 48k_r	AGCTCTTGATCCGGCAAACA	Minus	20	4781	4762	59.96	50.00	4.00	0.00

pAM-23K_f GCTGATATTCCCGCTGCTCT

COMPLEMENT 5'- AGA GCA GCG GGA ATA TCA GC -3'

pAM-23k_r AGAGCAGCGGGAATATCAGC

```
                          GCTGATATTCCCGCTGCTCT pAM-23k_f
  ctgccactgcttcgcgacctcgcgctgatattcccgctgctcttgtcgagtcagctttgg  < 4980
            4930       4940       4950       4960       4970
```

pAM-48k_r AGCTCTTGATCCGGCAAACA

COMPLEMENT 5'- TGT TTG CCG GAT CAA GAG CT -3'

pAM-48k_f TGTTTGCCGGATCAAGAGCT

```
                                                          TGTTTGCCGGATCAAGAGCT
  tctgcgcgtaatctgctgcttgcaaacaaaaaaaccaccgctaccagcggtggtttgttt  < 7260
            7210       7220       7230       7240       7250

  gccggatcaagagctaccaactctttttccgaaggtaactggcttcagcagagcgcagat  < 7320
            7270       7280       7290       7300       7310

                 TGTTTGCCGGATCAAGAGCT   pAM-48K_f
gctaccagcggtggtttgtttgccggatcaagagctaccaactctttttccgaa
7240       7250           7270       7280       7290
```

Forward primer (pAM54k_f)

```
                                GAGTGAGCTGATACCGCTCGCC     pAM27k_f n = 22 Tm = 61.9°C
tgattctgtggataaccgtattaccgcctttgagtgagctgataccgctcgccgcagccgaacgaccgagcgcagcgagt  < 160
            90         100        110        120        130        140        150
```

pAM54k_f: GAG TGA GCT GAT ACC GCT CGC C

Reverse primer (pAM54k_r)

```
                               {GAGTGAGCTGATACCGCTCGCC}     pAM27k_f n = 22 Tm = 61.9°C
tgattctgtggataaccgtattaccgcctttgagtgagctgataccgctcgccgcagccgaacgaccgagcgcagcgagt  < 160
            90         100        110        120        130        140        150
```

pAM54k_r: GGC GAG CGG TAT CAG CTC ACT C

Gibson primer design

For Gibson primers, fragments of the two different plasmids being joined must be combined into one primer that will overlap regions of the backbone and product vectors. The basic requirements here are that n ≥ 20 and T_m ≥ 50°C. For identification purposes, these primers will be labeled in an A-B_d fashion, where A is the whole fragment from one plasmid, B is the plasmid to which incomplete fragments of A are attached to the other whole fragment, and d is the direction (Figure 3).

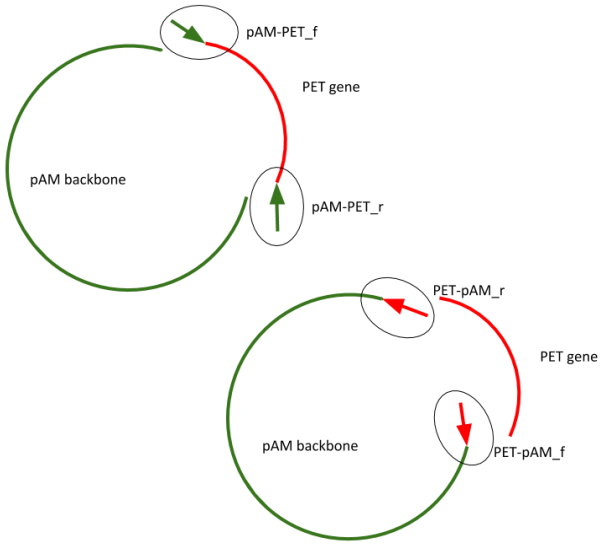

Figure 3. Schematic description of Gibson primer structure and naming convention.

PET-pAM primers

Forward primer

catctcagaagaggatctgtaa n = 22 T_m = 51.1°C
cggccaattgcagtgaacaaaaactcatctcagaagaggatctgtaatctagataggtaatctctgcttaaaagcacagaat < 1310
1230 1240 1250 1260 1270 1280 1290 1300

aagggtgggcgcgccgaccc n = 20 T_m = 70.7°C
aattgtacaaataaaagggtgggcgcgccgacccagctttcttgtacaaactcggccctcaggagaaggccatcctgacg < 2560
2490 2500 2510 2520 2530 2540 2550

catctcagaagaggatctgtaaaagggtgggcgcgccgaccc
…cggccaattgcagtgaacaaaaactcatctcagaagaggatctgtaaaagggtgggcgcgccgacccagctttcttgtacaaactcggccctcaggagaag…

PET-pAM_f: CAT CTC AGA AGA GGA TCT GTA AAA GGG TGG GCG CGC CGA CCC

Reverse primer

{atggcaaacaacgacctatt} n = 20 T_m = 52.4°C
catgaatcacaaagtgatggcaaacaacgacctattccaagccagtcgccgccgttttctggcacagctgggggggctca < 320
250 260 270 280 290 300 310

{ttaagaaggagcccttcacc} n = 20 T_m = 54.3°C
cctttgtttaactttaagaaggagcccttcaccatgtctaaaggtgaagaattattcactggtgttgtcccaattttggttgaattagatggtgat < 1840
1750 1760 1770 1780 1790 1800 1810 1820 1830

{ttaagaaggagcccttcaccatggcaaacaacgacctatt}
…cctttgtttaactttaagaaggagcccttcaccatggcaaacaacgacctattccaagccagtcgccgccgttttctggcacagctgggggggctca…

PET-pAM_r: AAT AGG TCG TTG TTT GCC ATG GTG AAG GGC TCC TTC TTA A

This was not quite done right, as all that was necessary was to take the complement of the PET-pAM_f primer to get the PET-pAM_r primer. In addition, an extra four base pairs were added to the 5' end of the original sequence to prevent primer-dimer potential, and then the complement was taken.

PET-pAM_r: GGG TCG GCG CGC CCA CCC TTT TAC AGA TCC TCT TCT GAG ATG AGT T

pAM-PET primers

Forward primer

```
                atggcaaacaacgacctatt  n = 20 Tm = 52.4°C
catgaatcacaaagtgatggcaaacaacgacctattccaagccagtcgccgccgttttctggcacagctgggggggctca  < 320
           250       260       270       280       290       300       310

            ttaagaaggagcccttcacc  n = 20 Tm = 54.3°C
ccttgtttaactttaagaaggagcccttcaccatgtctaaaggtgaagaattattcactggtgttgtcccaattttggttgaattagatggtgat  < 1840
    1750      1760      1770      1780      1790      1800      1810      1820      1830

            ttaagaaggagcccttcaccatggcaaacaacgacctatt
…ccttgtttaactttaagaaggagcccttcaccatggcaaacaacgacctattccaagccagtcgccgccgttttctggcacagctgggggggctca…
```

pAM-PET_f: TTA AGA AGG AGC CCT TCA CCA TGG CAA ACA ACG ACC TAT T

Original reverse primer

```
                        {catctcagaagaggatctgtaa}  n = 22 Tm = 51.1°C
cggccaattgcagtgaacaaaaactcatctcagaagaggatctgtaatctagataggtaatctctgcttaaaagcacagaat < 1310
1230      1240      1250      1260      1270      1280      1290      1300

             {aagggtgggcgcgccgaccc}  n = 20 Tm = 70.7°C
aattgtacaaataaaagggtgggcgcgccgacccagctttcttgtacaaactcggccctcaggagaaggccatcctgacg  < 2560
           2490      2500      2510      2520      2530      2540      2550

                          {catctcagaagaggatctgtaaaagggtgggcgcgccgaccc}
…cggccaattgcagtgaacaaaaactcatctcagaagaggatctgtaaaagggtgggcgcgccgacccagctttcttgtacaaactcggccctcaggagaag…
```

pAM-PET_r: GGG TCG GCG CGC CCA CCC TTT TAC AGA TCC TCT TCT GAG ATG

Again, the complement of the pAM-PET_f primer should have been taken for the pAM-PET_r primer. However, to ensure minimal primer-dimer potential an extra three base pairs were added to the 5' end of the original sequence before taking the complement

pAM-PET_r: AAT AGG TCG TTG TTT GCC ATG GTG AAG GGC TCC TTC TTA AAG

Internal PETase primers

Strategy

We do initial screening with the sequencing primers that flack the gene insertion site in the vector backbone. Every plasmid backbone has a _f and_r sequencing primer which is used to sequencing interred genes, and can also be used to PCR the interest up. When screening by PCR we look for a specific size (size of insert plus flanking sequence) . However this does not provide specific amplification due to the gene, and might be misleading if there is an insert of similar size.

To confirm OUR gene is inserted, we want a specific primer, which will ONLY amplify our gene of interest. This will be a lot more diagnostic. We can do this by making primers specific to the gene, and using one of these as the _f or _r primers in conjunction with the sequencing _r an d _f primers.

PETec-N_r1

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	CATCGGTGATGTCGGCGATATAGG	Plus	24	286	309	63.01	54.17	7.00	2.00
Reverse primer	CGGACCCCACCACTTAATGC	Minus	20	777	758	61.03	60.00	4.00	2.00
Product length	492								

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Primer pair 6

PETec-N_r2

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity		Self 3' complementarity
Forward primer	CATCGGTGATGTCGGCGATATAGG	Plus	24	286	309	63.01	54.17	7.00	2.00	
Reverse primer	GTGTACCCGGGGACGATTG	Minus	19	743	725	60.15	63.16	8.00	2.00	
Product length	458									

PETse-int-C_f

Primer pair 1

Sequence (5'->3')	*Template strand*	*Length*	*Start*	*Stop*	*Tm*	*GC%*	*Self complementarity*	*Self 3' complementarity*
Forward primer **CAGTTCCGTCACAGTTCCCA**	Plus	20	761	780	59.89	55.00	3.00	0.00
Reverse primer CTTCAAAAAGGCCATCCGTC	Minus	20	1173	1154	57.36	50.00	4.00	1.00
Product length 413								

PETse-int-N_r

Primer pair 1

Sequence (5'->3')	*Template strand*	*Length*	*Start*	*Stop*	*Tm*	*GC%*	*Self complementarity*	*Self 3' complementarity*
Forward primer TCATCGGCTCGTATAATGGT	Plus	20	1	20	56.22	45.00	4.00	0.00
Reverse primer **CACAATTGCGATAGCACCGA**	Minus	20	440	421	58.99	50.00	6.00	0.00
Product length 440								

pETec_C-f3

agtttctggaaattaacggcggt n=23 T_m =57.2°C

aacagtttctggaaattaacggcggtagccactcttgtgccaactctgggaacagcaaccaggcactgatcgga

5880 5890 **5900** **5910**

pETec_C-f4

cttcaaccaacttcagcagtg n=22 T_m =55.7°C

cgccatgggactcttcaaccaacttcagcagtgttaccgtgccgacgctgatt

GFP-N_r

tgtttttctagatacccagatcatatgaaacaaca

{tgtttttctagatacccagatcatatgaaacaaca} n=35 Tm=57.9 C

ttcaatgtttttctagatacccagatcatatgaaacaacatgacttttcaagtctgccatgcc

1910 1920 1930 1940 1950 1960 1970 1980 1990

complement: TGT TGT TTC ATA TGA TCT GGG TAT CTA GAA AAA CA

Primer (**PCR**, *Gibson*)	Sequence
Fragmentation	
pCS-PET_f	ATG GCA AAC AAC GAC CTA TTC C
pCS-PET_r	TTA CAG ATC CTC TTC TGA GAT GAG TT
pAM_f	AAG GGT GGG CGC GCC GAC
pAM_f2	AAGGGTGGGCGCGCCGACCCAGCTTT

pAM_r	GGT GAA GGG CTC CTT CTT AAA G
PET-pAM_f	*CAT CTC AGA AGA GGA TCT GTA AAA GGG TGG GCG CGC CGA CCC*
PET-pAM_r	*AAT AGG TCG TTG TTT GCC ATG GTG AAG GGC TCC TTC TTA A*
pAM-PET_f	*TTA AGA AGG AGC CCT TCA CCA TGG CAA ACA ACG ACC TAT T*
pAM-PET_r	*GGG TCG GCG CGC CCA CCC TTT TAC AGA TCC TCT TCT GAG ATG*
pAM35k_f	CGA ATT GAT CGG CAA GCC AG
pAM35k_r	CTG GCT TGC CGA TCA ATT CG
pAM27k_f	GCA GGT ACA TGG GCC AGC AT
pAM27k_r	ATG CTG GCC CAT GTA CCT GC
pAM23k_f	GCTGATATTCCCGCTGCTCT
pAM23k_r	AGAGCAGCGGGAATATCAGC
pAM48k_f	TGTTTGCCGGATCAAGAGCT
pAM48k_r	AGCTCTTGATCCGGCAAACA
pAM54k_f	GAG TGA GCT GAT ACC GCT CGC C
pAM54k_r	GGC GAG CGG TAT CAG CTC ACT C
Screening	
PETec-N_r1	CGGACCCCACCACTTAATGC
PETec-N_r2	GTGTACCCGGGGACGATTG
PETse-int-N_r	CACAATTGCGATAGCACCGA
PETse-int-C_f	CAGTTCCGTCACAGTTCCCA
pETec_C-f3	agtttctggaaattaacggcggt
pETec_C-f4	cttcaaccaacttcagcagtg
GFP-N_r	TGT TGT TTC ATA TGA TCT GGG TAT CTA GAA AAA CA

(above) Master table of primers used in experiments.
(below) Master table of fragment names with corresponding primers and size. Highlighted are the fragments used to put together pAM-PET.

Name	Template	Forward Primer	Reverse Primer	Size (bp)
PET	pCS_PET	pAM_PET_f	PET_pAM_r	1034
Frag A	pAM	pAM_F2	pAM_35k_r	3521
Frag B	pAM	pAM_35k_f	pAM_r	3554
Frag C	pAM	pAM_F2	pAM_23k_r	2468
Frag D	pAM	pAM_23k_f	pAM_48k_r	2330
Frag D’	pAM	pAM35k_f	pAM48k_r	1278
Frag E	pAM	pAM48k_f	pAM_r	2295

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