Jasmine's Lab Notebook

Day 1: Designing Primers 6/8/15

Forward primers

vial has 38.2 nmol x 10= 380 µL water added to vial

(This created a 100 µM solution (stock)).

Took 1 vial dilution --> 2.5 µL from stock (used for promoter PCR reaction)

What are we doing?

```
            Designing primers
```

Why?

```
            "Good primer design is essential for a successful PCR reaction."
```

Methods *(Using APE Program)*

1) In general, a length of 18-30 nucleotides for primers is good.

2) Try to make the melting temp of the primers between 55°C and 70°C, and within 5°C of each other.

3) If the melting temp of your primer is very low, try to find a sequence with more GC content, or extend the length of the primer (minimally).

4) Aim for GC content to be between 40 - 60 %, with the 3' of the primer ending in G or C to promote binding.

5) 3-4 nucleotides are added to 5' of the restriction enzyme site in the primer to allow for efficient cutting.

6) Try to avoid regions of secondary structure *(check by using the OligoCalculator)*, and have a balanced distribution of GC-rich and AT-rich domains.

7) Try to avoid runs of 4 or more of one base, or dinucleotide repeats *(ACCCC or ATATATATATAT)*.

8) Avoid intra-primer homology *(more than 3 bases that complement within the primer)* Avoid inter-primer homology *(forward and reverse primers having complimentary sequences)*.

(Otherwise, these can lead to self-dimers or primer-dimers instead of annealing to the desired DNA sequences).

PCR ran for constitutive promoters

Amplify your promoter variants and BAR1 with PCR, usings reactions to add restriction enzyme cut sites so that the pieces can be inserted into the plasmid backbone via ligation. (2 PCR reactions per group).

Reagents used

Promoter PCR Reaction:

1. Forward primer (10 µM) 2.5 µL
2. Reverse primer (10 µM) 2.5 µL
3. Template DNA 0.5 µL
4. 2x Phusion Master Mix 25 µL
5. Water 19.5µL

```
Total 50 µL
```

Bar1 PCR Reaction:

1. Primer #92 (10 µM) 2.5 µL
2. Primer #93 (10 µM) 2.5 µL
3. Bar1 Template DNA 1 µL
4. 2x Phusion Master Mix 25 µL
5. Water 19 µL

```
Total 50 µL
```

Note: Your stock primers are 100 µM. You must make a tube of diluted primer to use for cloning.

Next

1. Mix the above reactions in PCR tubes on ice. Make sure to mix well since the enzyme is viscous and sinks to the bottom.
2. Put in the thermocycler for the following cycle:

```
- Initial Denaturation     98°C  30s
    - 35 cycles of
            - Denaturation 98°C  10s
            - Annealing    55°C  20s
            - Extension    72°C  1m

- Final Extension          72°C  5m
- Hold                      4°C  forever
```

3. Keep samples for gel extraction the next day.

Day 2: Enzyme Digestion 6/9/15

Our plasmid was designed to have different cut sites between each fragment of interest.

Plasmid= pjw608

Endonucleases= Apa1, Xho1, and Not1

We set up diffferent digestion reactions for each part of the DNA.

Promoter Digest:

```
1. Add 5 µL Cutsmart buffer to your PCR tube
2. Add 0.5 µL Apa1 to your tube. Vortex to mix well.
3. Incubate at room temp for at least 1 hour.
4. (Stop)
5. Add 0.5 µL Xho1 to the tube. Vortex to mix well.
6. Incubate at 37°C for at least 1 hour.
```

Bar1 digest:

```
1. Add 5 µL Cutsmart Buffer to your PCR tube.
2. Add 0.5 µL Xho1 and 0.5 µL Not1 to the tube.
3. Vortex to mix well.
4. Incubate at 37°C for at least 1 hour.
```

Plasmid Digest:

```
1. Take 10 µL of plasmid from the plasmid stock (pjw608) into a new tube.
2. Add 3 µL CutSmart Buffer
3. 16 µL of water to your tube.
4. Incubate at room temp for at least 1 hour.
5. (Stop)
6. Add 0.5µL Not1 to the tube. Vortex to mix well.
7. Incubate at 37°C for at least 1 hour.
```

Point: Done to amplify promoter variants (strains) and Bar1 via PCR.

- Rxns used to insert restriction enzyme cut sites to anneal pieces into plasmid backbone via ligation.
- Transformation done; Colony PCR next.

Day 3: Colony PCR 6/10/15

Ideally, we want to make Xho1 + Bar1 (in forward direction) and Bar1 + Not1 (in opposite direction).

We were assigned pTEF m10 mutant strain. (other mutant strains exist --> pTEF1 (m1, 3, 6, 7, 10)

Results from Colony PCR (Via Gel Electrophoresis)

Team data (NJ) on lanes 12-15 (GEL #1).

```
- 12: Promoter
- 13: Promoter
```

```
- 14: Bar1
- 14: Bar1
```

Team data (NJ) on lane 7 (GEL #2).

```
-7: P1 plasmid
```

Took Nanodrop of colonies. Took 2 cell colonies (5 µL plasmid samples).

```
Named NJ1 and NJ2
NJ1= Cell colony 1
NJ2= Cell colony 2
```

Results of Nanodrop:

```
- (Plate #1)
- Promoter 600.0 ng/µL
- Promoter 635.1 ng/µL
- Bar1     618.6 ng/µL
- Bar1     353.5 ng/µL

*Bar1 with lower concentration is residual from first Bar1


- (plate #2)
- Plasmid 543.6 ng/µL
- Plasmid 49.96 ng/µL
- Bar1    14.18 ng/µL
- Prom.   117.3 ng/µL
```

Day 4: Ligation 6/11/15

Now, we need to combine the pieces that we've made.

(We need the same cut sites so that the primers, template, and plasmid can all anneal.

New Protocol:

1. Cut gel slides
2. Protocol- QIAGEN QIAQUICK Gel Extraction
3. Elute (w/water 30 µL)
4. Take DNA concentration using Nanodrop (1.5 µL)
5. Calculate amounts of DNA to ligate together.

Volumes used:

```
          - 600 µL promoter/temp DNA
```

```
        - 450 µL Bar1
        - 470 µL plasmid
```

1. 2 µL DNA ligase buffer
2. 1 µL insert DNA
3. 5.5 µL Bar1
4. 1 µL DNA ligase (Add last)

(*Total needs to be 20 µL; dilute the rest with water* 10 µL water in this case)

Workshop/ Lecture Days 6/ 12-17 /15

(Little to no lab work done during these dates)

Day 9: Gel Extraction and Digestion 6/18/15

We checked our ligation by running a gel.

After Gel Extraction and digestion, we waited days for primers and borrowed sequencing data.

1st try: (Bands not where we wanted them to be, so we did it again using 50 µL Phusion Protocol.

- LexADBD FW --> 126 --> 24.8 nmol = 248 µL water needed
- LexADBD RV --> 127 --> 31.2 nmol = 312 µL water needed
- pFIG2C FW --> 128 --> 31 nmol = 310 µL water needed

(water added to make working stock, otherwise primers are too concentrated to work with).

2nd try: bands were not where they were supposed to be; redo gel.

3rd try: no bands; redo gel; change protocol.

```
    Protocol Change--> take PCR product from before and PCR them as the template.
```

Day 10: Another Workshop/Lecture Day 6/19/15

(Little to no work done today).

Day 11: Digest and PCR 6/24/15

Protocol Alteration: Digest: Gel Extract --> Pour Gel and load all DNA elute in 30 uL water and nanodrop.

PCR: Use new Td_tomato template (Blue label, 0.5 uL) Also, do one set with and without DMSO

```
Increase annealing temp to 60°C;
Extension time to 1.5 min (1:30 mins).
```

```
What we want: Plasmid top bad; pAga1+ mCherry to be cut out (piece below the plasmid in same lane); we also want a 2.5 kb PCR product.
```

Results: We see bands! (we took middle 2 bands. Both are LexA. One with DMSO and one without DMSO).

```
    w/DMSO 2nd lane and w/out DMSO 3rd lane.
    Ladder 1st lane
```

Day 12: Plasmid Digests and Gel Extraction 6/25/15

Plasmid Digests: (Testing using 2 different plasmids).

```
       Xho1 + Not1            Apa1 + Not1
       (w/ Hy130E)            (w/ Hy130E)
       (w/ PGEM 22)           (w/ PGEM 22)
```

Digest Gel Purification, then run nanodrop.

Gel Purification (Plasmid)

```
10 µL Hy130E into a new tube
3 µL cutsmart buffer
16 µL water
0.5 µL Apa1 or Xho1 (depending on form achieving)
Vortex.
Incubate @ room temp for ~1hr
Add 0.5 µL Not1 (to both tubes).
Vortex
Incubate @37°C ~1hr.
```

Nanodrop Results from Gel Purification:

```
LexA                   pFig2C
23.97 ng/µL            13.53 (ng/µL)
```

Note: pGEM22 did not work (no separation = sequence chosen was incorrect)

Hy130E was able to digest successfully and separate.

For this reason, we went on to use Hy130E extracts.

Nanodrop Results:

```
    (Hy130E +Apa1 +Not1)           39.76 ng/µL

    (Hy130E +Xho1 +Not1)           44.13 ng/µL
```

Gel Extraction of LexA and pFig

```
1st tube --> LexA gel extraction
         --> 231 mg (no promoter)

2nd tube --> pFig gel
         --> 6 mg (troublesome about small weight)

Add 3 volumes of buffer QG per 1 volume of gel
(100mg to 100mL, etc.)

Buffer needed: 1st tube --> 693 µL
               2nd tube --> 18  µL

Incubate @ 50°C for 10 mins;
Mix every 2-3 mins

Add 1 gel volume of isopropanol to the sample & mix.
(1:1 ratio)

            1st tube --> 230 µL iso added
            2nd tube --> 6   µL iso added
```

Day 13: Transformation 6/26/15

Performing Transformation (High Efficiency protocol)

C2987 cells used.

```
*Thaw on ice for 10 mins
*25 µL of cells into a transformation tube on ice
*1-5 µL with 1pg-100ng of plasmid DNA to cell mix
*flick 4-5 times
*Do not vortex
*place on ice for 30 mins
*Do not mix
*place on ice for 5 mins
*Do not mix
*Pipette 950 µL of room temp SOC media into mix
*Place @ 37°C for 60 mins
*Shake vigorously (250 rpm) or rotate.
*warm selection plates to 37°C
*Mix the cells thoroughly using via flicking tube & inverting
*Perform several 10-fold serial dilution in soc
*spread 50-100 µL of each dilution onto a selection plate and incubate overnight @ 37°C
(or 30°C overnight for 24-36 hrs; 25°C for 48 hrs).
```

Day 13: Colony PCR 6/29/15

Colony PCR Performed

Alterations: 3min extension time @ 72°C 6 colonies from 8x plate pNH604 +LexA +pFig

```
(To reduce background self-ligation, add 1µL SAP to digested plasmid)
```

Day 14: Gel of Colony PCR 6/30/15

Ran Gel of Colony PCR results to see if bands appear where we need them.

```
Gel showed that colony PCR results from Hy130E +xho1  +Not1 +LexA 8x worked

Colony PCR for Hy130E +Apa1 +Not1 +pFig 8x did not work.
```

Then we reperformed ligation w/SAP treated plasmid on Hy130E +Apa1 +Not1 cut sites.

Redoing colony PCR from Hy130E +Apa1 +Not1 and performing liquid culture.

Liquid Culture

```
5 mL LB +CARB media/ falcon tube.
put all of the colony PCR tube in each falcon tube
(most likely 20 µL)
Let sit in 37°C room on shaker overnight.
(USE TUBE HOLDER)!!!
```

Results from colony PCR (Gel)

Ran:

```
Hy130E xho1 +Not1 +LexA 8x +pFig +pNH604

&

Hy130E Apa1 +Not1 +LexA +pNH604
```

Results: 5 colonies worked for Hy130E xho1 +Not1 +LexA 8x +pFig +pNH604; however, no colonies worked/ no bands for Hy130E Apa1 +Not1 +LexA +pNH604 occurred.

Ligation is performed as a backup just in case the band does not show where we need it to (we need separation).

No bands present means that the template strand that we are using is not annealing to the DNA that we are trying to PCR (amplify).

Possible that other colonies have our DNA that we inserted, just need to find other colonies to test (to find our DNA where it took).

Afterwards, we ran a 2nd colony PCR. only colony 8 worked (1st & last lanes were ladder)

Colony 8= Hy130E xho1 +Not1 +LexA 8x +pFig +pNH604

liquid culture of successful colonies from PCR to shake overnight.

Checked later in the day, some tubes fell off of shaker, colonies 1 & 4 (from ligation backup) lost.

Day 15: Miniprep of Liquid Culture 7/1/15

Minipreped Colony 8, and also back up ligations that did not break (some tubes broken and fell off of shaker from ligation backup).

Colonies minipreped from ligation: 2, 3, 5, and 6

Samples chosen for sequencing: 2 & 8 (not labeled with these numbers on sequencing order).

Sent to sequencing 10:19 am.

Note: HyA= pFig2c + LexA +pNH604 w/ Apa1 +Not1 cutsites.

Miniprep sequencing numbers:

```
HyA C8 --> #1
HyX C5 --> #2
HyX C6 --> #3
HyX C2 --> #4
```

Nanodrop Results:

```
#1 739.6 ng/µL
#2 474.0 ng/µL
#3 699.6 ng/µL
#4 549.9 ng/µL
```

Sequencing done: #'s: 1, 2, & 4.

Day 16: Colony PCR Gel + Sequencing Results 7/2/15

Sequencing Results:

```
#1 worked.
    Our plasmid was there, still need to double check if our LexA DBD is there as well.

#2 & 4 Did not work.
```

```
    Our DNA was not present.
```

Solution: Start another colony PCR (used Hy130E (+xho1 +Not1 Cut sites) + LexA DBD part 8x) Chose 8 colonies.

Gel Running: Colony PCR #3 Hy "x"

Lanes:

1. 10 µL Ladder
2. space
3. Hy"x" colonies (#'s 1-6)
4. "
5. "
6. "
7. "
8. "
9. overspill of colonies (#'s 7-8)
10. 10 µL Ladder
11. colony #7
12. colony #8

Results: Colony 8 (called #1) had lexA DBD (it worked) However, we had incomplete coverage Therefore, we sent it again with new primer.

```
Colonies 2 & 5 have no insert of LexA DBD (TF).
```

Primers came.

1. Reconstitute primers with water like normal.
    1. read nmol weight and multiply by 10 for the µL water needed to be added.
2. Instead of making 10-fold dilution, take 50 µL of diluted primer & 950 µL water.
    1. Label tube "pNH604F"
3. Put the tube with primer in with your sequence reaction (5 µL #1).
4. Put in sequence box.
5. Plasmid size= 8 kb
6. 3 µM conc.
7. we sent a stock of pNH604F primer also.
8. Also, we did a liquid culture.

Ran a 3rd Colony PCR (w/ new colonies from Hy130E +Xho1 +Not1 8x plate).

```
Lanes 3-7 = colonies 1-6 in order

Well between lanes 8 & 9 fell over.
```

```
We reloaded colonies 7 & 8 into lanes 11 & 12.
```

Also performed a simple miniprep of colonies: 1, 2, 7, & 8 from Hy130E +Xho1 +Not1 8x plate

Nanodrop Results:

```
Hy130E "x"
    1--> 643.9 ng/µL
    2--> 699.7 ng/µL
    7--> 748.4 ng/µL
    8--> 415.5 ng/µL
```

Day 17: Re-digest & Sequencing 7/6/15

Redo digests Redigest--> Apa1 for a couple hours for 1st step --> add Not1 for overnight treat also in other tube, xho1 + Not1 endonucleases to sit overnight as well.

Send off 2 minipreps for sequencing.

Sent off: 2 & 7

Tube with orange label --> HyA +Apa1 +Not1 (Already had cut sites; accident)

Tube with green label --> * Hy130E original plasmid with no cut sites. * (Apa1 +Not1 added)

Tube with no label --> * Hy130E original plasmid with no cut sites. * (Xho1 +Not1 added)

Sequencing of pFig2c-lexA showed additional DNA fragment of STE2 receptor (Blasted DNA sequencing results to arrive at this conclusion).

As a result, we needed to redigest to completion

New sequence of colonies.

Day 17: Phosphotase Treat + Gel Ran 7/7/15

Phosphatase Treat miniprep'd tubes

Phosophatase Treating Procedure:

```
* 0.5 µL phosphotase (Antartic phosphotase)
* Incubate @ 37°C for 1 hr.
* Incubate @ 65°C for 10 mins.
```

Pour a gel --> made TAE buffer dilution Gel extract Purified bands.

Gel photo lanes:

```
ladder
skip
HyX (no prior cutsite)
skip
HyA new (no prior cutsites)
skip
HyA (cut twice)
```

Left all tubes in 37°C room all night.

Gel shows that the enzymes did not cut.

Assumption: maybe the enzymes are dead. other teams were having problems with this also.

Solution: replaced enzymes re-digest with new enzymes.

Gel extraction for this reaction was not able to occur.

Waited a long time for other people's gels and for weight room to be free for use to make agarose 1%.

Day 18: Gel Extraction?? 7/8/15

Attempted to Gel extract results, however Gel shattered.

As a result, no image available.

Assumption: Bad agarose. Other groups had problems with gels shattering as well.

Solution: threw out agarose and made some more.

Since it was a gel extraction, we had no more sample to use (entire sample administered).

(inefficient to gel extract our plasmid and redo the procedure); this causes higher loss of product (DNA & Plasmid).

As a result, we redid the entire procedure starting with plasmid digestion protocol with Hy130E plasmid.

Digestion will run overnight.

Separately, I also ran a transformation on Hy130E with cells C2987. We were running out of Hy130E.

Tomorrow, we hope to run a ligation on digest materials.

Day 19: Gel of Digestion Products 7/9/15

Pour and ran a gel of yesterday's digestion.

Lanes:

```
ladder
```

```
skip
HyA
HyA
skip
HyX
HyX
ladder
```

Weights: HyA --> 214 mg HyX --> 257 mg

Then performed gel extraction procedure.

Calculations:

```
    HyA --> 214 mg x3 = 642 µL Buffer QG
     actual amt added = 640 µL

    HyX --> 257 mg x3 = 771 µL Buffer QG
     actual amt added = 770 µL
```

(Remember, Buffer QG is 3:1 volume).

After incubation @ 50°C for 10 mins, add 1 gel volume isopropanol.

```
    HyA --> 214 mg
    actual amt added = 210 µL

    HyX --> 257 mg
    actual amt added = 260 µL
```

(See rest of directions protocol: QIAquick Gel Extraction kit using microcentrifuge).

Gel Results from digestion of HyX & HyA

Gel shows that the plasmid actually digested!

```
    --> Success!
    --> Top band was extracted
    --> Lane identies above
```

Nanodrop Results:

```
    HyA --> 97.15 ng/µL

    HyX --> 76.54 ng/µL
```

Next, we performed ligation. --> Used NE Bio Calculator to calculate molar ratios needed.

```
Insert DNA Length --> 2 kb          (HyX)

Vector DNA Length --> 8 kb

Vector DNA Mass   --> 76.54 kb      (HyX)


3:1 Ratio gives   --> 57.41 ng      (HyX)
```

LexA concentration --> 23.97 ng/µL

```
               --> 2 µL LexA used.
```

pFig concentration --> 13.53 ng/µL

```
               --> 7 µL pFig used.


Insert DNA Length --> 2.8 kb            (HyA)

Vector DNA Length --> 8 kb

Vector DNA Mass   --> 76.54 kb      (HyA)


3:1 Ratio gives   --> 102.0 ng      (HyA)
```

(See ligation protocol w/T4 DNA Ligase).

Left for 2 hours.

Transformation done afterwards and plated them for colony growth.

(4 tubes done --> negative control ran for each tube).

Note: Negative control means no LexA added. (Extra water added to maintain volume).

Day 20: Colony PCR + Liquid Culture 7/10/15

Colony PCR of yesterday's transformation.

```
--> attempted: however, very little colonies.

--> Solution: Put plates in 37°C Room for a few more    hours.
```

Meantime, liquid culture, then miniprep transformation of new plasmid Hy130E that was remade (from stock).

Liquid Culture

```
    --> 5mL LB + CARB media in falcon tubes
        put colony in falcon tube.
        set in 37°C room on shaker overnight.
```

After a few more hours, not many more colonies formed.

```
    -->Solution: 1) Colony PCR HyA (this plate had a               few colonies).

                 2) Redo ligations of HyX (these    plates had no colonies).
```

Results of Colony PCR Ran 1.5% agarose gel.

```
    Explains the smudging of ladder (This gel was different than normal; required to run at a lower current);
(75V instead of 100V).

    Very flimsy gel.
```

Day **: Saturday Miniprep 7/11/15

Miniprep'd New Hy130E plasmid (uncut) from liquid culture state.

Day **: Sunday Transformation of Ligation 7/12/15

Transformation of ligation HyX.

Held in fridge prior to transformation.

Day 20: Transformation Check + PCR of Inserts 7/13/15

Checked transformation --> no colonies --> do process again.

1) Reran previous colony PCR products on gel to check cut sites also creating new ones with fresh enzyme for comparison.

2) PCR inserts to amplify before we run out.

Day 21: Colony PCR Gel + Gel Extraction 7/14/15

Poured & Ran a gel from Colony PCR results.

```
--> Bands resulting were way too high (in previous gel ran)
    this is why we tried comparing both old digest and new primers as templates.
```

```
--> Start again with digestion.
```

Nanodrop Results:

```
LexA1 --> Old digest as template
      -->  377.8 ng/µL

LexA2 --> New Digest as template
      --> 414.0 ng/µL

pFig1 --> Old digest as template
      --> 452.2 ng/µL

pFig2 --> New Digest as template
      --> 446.3 ng/µL
```

Note: Xho1 does not have pFig2c. Apa1 has pFig2c.

Now we are digesting the PCR Results.

Poured Gel and performed Gel Extraction Protocol.

Weights:

```
    LexA Gel:

            --> 147 mg
            --> used 440 µL QG Buffer
            --> used 150 µL Isopropanol
            --> the rest is the same.

    pFig Gel:

            --> 228 mg
            --> used 690 µL QG Buffer
            --> used 230 µL Isopropanol
            --> the rest is the same.
```

We also performed a ligation and transformation.

Nanodrop Results of inserts:

```
    L1 (LexA) --> 23.65 ng/µL

    F1 (pFig) --> 16.49 ng/µL
```

PCR of inserts Lanes:

between 1st and 2nd ladders: Josh's samples.

After 2nd ladder: our samples.

```
    --> 2nd ladder
        skip
        F1
        F2
        L1
        L2
```

Gel Extraction of L1 and F1

Lanes:

```
ladder
skip
F1
F1
skip
L1
L1
```

Gel showed bands were we needed them so we proceeded with Gel Extraction.

Day 22: Another Colony PCR + Liquid Culture 7/15/15

1) No colonies at first, waited a few more hours to validate results of transformation.

After a few hours,

```
    8x HyX +L1
    8x HyA +F1

    The colonies from these plates were used for colony PCR.
    The other dishes were thrown out.

    The other dishes did not have nearly as many colonies; not as promising.
```

2) Set up Colony PCR

3) Also, set up a liquid culture to grow the cells in the colony.

Day 23: Gel for Colony PCR + Miniprep 7/16/15

Ran gel for Colony PCR of (8x HyX +L1) & (8x HyA +F1) colonies.

Split Gel into half:

```
        --> 8 colonies on top: (8x HyA +F1)

                        and

        --> 8 colonies on bottom: (8x HyX +L1)
```

Miniprep'd & Nanodrop'd

Miniprep'd:

```
        (8x HyA +F1) --> Colonies 2 & 6.

        (8x HyX +L1) --> Colonies 1, 2, 4, & 5.
```