3rd September:

Miniprepped 30 samples (4 of the B batch) 2 of xenia's and 24 of 3x colony pickings over 8 plates (4 different CBDs)

Sent stuff in to sequence (4 of the miniprepped) 2 of xenia's and 2 of laura's stuff.

Analysed the sequences to confirm Freiburg sites and that they are the clones that we think they are.

Plan back insert, front digestions using online excel that Xenia made

4th September 14

PCR – T7 expression biobrick

Digest sfGFP from 5ng tube. And do test digest when it is in backbone!

Did digest with the front and back inserts of CBD (based on the orientation of the linker)

5th September 14

Poured gel and ran all of our digestion product on the gel.

Gel extracted the bands and nanodropped. (nanodrops are quite low but we will still use it for the digestion).

Did some lab admin and filled up 4 tubs of 1.5ml eppendorfs for general use.

Nanodropped the T7 PCR products as well.

Running the PCR products on the gel too but didn't see anything.

Poured over 120 CAM plates, left them to dry overnight.

6th of September:

Packed the CAM plates from last night and moved them to the cold room. Moved the other biobrick plates to the cold room too.

Ran the gel with the PCR product again, and will view under the transilluminator at Jeff's lab. Didn't see any bands.

Did transformations on competent cells with the confirmed sequences (there were 9. Xenia plated these, and Ben moved the plates to the fridge.

8th September

Planned test digest for the CBDs and asked for some enzymes from Ben.
Set up 9 liquid miniprep cultures to do this. Made the plate stocks! (Split the plate into 4 and dabbed the one colony that is used for setting up miniprep liquid samples.
Ran the PCR again with T7 Promotor overnight, but with/without DMSO, and with either GC buffer or HF buffer.

9th of September

Poured 7 gels to prepare for the test digests. (Xenia took 2 big gels, we used one gel to run the PCR T7 promotor, and then 4 big gels for the test digests)
Ran the PCR for T7 promotor on gel.
Took picture of them at Jeffs lab.

Did the test digests for 24 of the CBDs in backbone picked colonies.
Loaded the test digests on the gel and Ran this gel. Took photos and sent via email.
Miniprepped the samples from yesterday (9samples) and nanodropped these.

Digested the sfGFP and T7 promotor biobrick plasmid into linear (to make the sfGFP in backbone, and T7 promotor as a digestible plasmid.

Redigested the CIPa CBD with XbaI and PstI, and also backbone with XbaI and PstI. (to repeat CipA in backbone cloning)

Pool the 4 samples and DpnI the T7 promotor PCR products. And PCR purify them.
PCR purify the sfGFP and the T7 promotor templates, and the other digests (CipA and Backbone).

10th September:

Removed the stock plates with colonies, from incubator to fridge.
PCR purified the T7 vector that was DpnI overnight.

Nanodropped the T7 vector and the linearized sfGFP and new T7. Also the Cip A and its backbone pair that were both digested.

Set up the PCR for the sfGFP into Freiburg format, as well as the fmT into Freiburg. Will finish around 3.15pm

Pour 2 gels. Ran the sfGFP and fmt pcr products on the gel. 1.5 hours.
And 3ul of the pcr purified of the T7 promotor to gel.

Add kinase to the T7 promotor vector.
Half an hour with the kinase.

<u>Phosphorylation of the ends with kinase [15ul reaction volume]:</u>
10ul of the Pcr purified/gel extracted T7 vector DNA
2ul of T4 ligase buffer
2.5ul water
0.5 T4 polynucleotide kinase

[Self-ligate blunt ends of T7 vector]
<u>Overnight ligation:</u>
1ul, 3ul and 5ul phosphorylated DNA (set up 3 different tubes for ligations)
7.5ul, 5.5ul and 3.5ul water
1.0ul T4 ligase buffer
0.5ul T4 ligase

Made Glycerol stocks and labeled them with the red and green system. Also set up a new box for glycerol stocks in the -80 freezer.

And then DpnI the pooled sfGFP and fmT PCR products for half and hour, and PCR purified this.

Digested the sfGFP, fmT, and the backbone with Xba and PstI (like CipA, so we can put it into the same backbone that was digested, except that we realized we didn't dephosphorylate the backbone digestion (to prevent it from recircularising).

Ligated the phosphorylated T7 DNA overnight.

Set up overnight PCR with T7 linearised template.

11th September

Pour a gel to test the T7 promotor pcr with linear template.

Ran the gel. And took images. The bands looked good for all except for R2, which was HF buffer with DMSO. Brighter bands with GC buffer as well.

Dephosphorylated the backbone 16, using the below recipe:
Onto the 20ul digest reaction, added:
2.5ul Antarctic phosphatase buffer,
1.25ul of Roche Rapid Phosphatase,
1.25ul of water.
-To make a total of a 25ul phosphatase reaction, which we let run for more than half an hour before heat inactivating at 72C for 10 minutes.

We PCR purified the phosphatased backbone 16 and sfGFP and fmT and got very low nanodrops for backbone 16. We are unsure why this would be, but still couldn't use this for the ligations with sfGFP, fmT and CipA, so instead, we set up 6 new digestions of the backbone, using the CBD CipA in backbone that seemed good as ligations (we analysed the gel results for the CBDs on the test digest for the ones we can save) onto the backbone but had been cut at an illegal cut site.
Half of these digestions will be gel extracted, the other half PCR purified. With each trio, we will try doing single adsorption through the column, and double, then triple.

Ran all the T7 promotor pcr (batch 2) in the gel and gel extracted.

This gel extracted T7 vector DNA needs to be treated with kinase, same as the first batch and will be left to self ligate tomorrow.

Analysis of the gel results for the CBDs for the ones we can use, we found that all of CBD 1 had the same pattern but not one we'd expect, all of CBD2 was confirmed as good. CBD10 (CipA had 2/3 without the smaller cut, which was good. The rest we would use for backbone template rather than throw away (as they had very high concentrations). CBD15 was all confirmed.

We threw away lots of the cloning intermediates (digests/ligations/minipreps) in the freezer of samples that either did not have the correct test digest pattern, or were confirmed via sequencing as being failed clonings.

12th September

The gel extracted fragments were treated with kinase, and are left to ligate for a few hours. (We did ligations of 1ul, 3ul, and 5ul for each of the 4 tubes of kinase treated DNA, therefore we had 12 ligation reactions). The four tubes correspond to the 4 PCR tubes that were all run on the gel in their own wells.

Send the new CBDs confirmed by test digest from yesterday to sequencing at 3pm.

Set up PCR reaction for Xenia.

3 of the digests were gel purified and the other 3 were PCR purified.

The 3 of the digests that were run on the gel didn't end up being gel purified as they did not display the correct bands. All three showed up as a distinct 2800 band, which suggests that one of the enzymes had cut but not the other (single cut gives the same as the size of the template plasmid but not blurry/amorphous on the gel).
We will run a little bit of the 3 T7 DNA vector that was PCR purified on the gel to see if any of them were digested correctly.

In order to deduce which enzymes are/is cutting well and which ones aren't/isn't, we decided to do another digest test with the same failed CBD and the backbone 16 that we had previously used successfully, to use do the following 8 diagnostic digests (these are all using cutsmart with the red dot):

- Xba + PstI
- Xba alone
- PstI alone
- Neither

We will also try a set of the above 4 treatments with Backbone 16 and with the 'blue dotted cutsmart' from yesterday, and tube 13 (the same exact failed CBD10 that was used for those earlier backbone reaction).
Bands from these digests will let us know whether it was the template we used that was poor, if it was either/both of the enzymes, or if it was all the cutsmart buffer that was used.

Transformed tonight x 4 of the T7 promotor ligations (1ul, 3ul and 5ul of Reaction 3, and 3ul of Reaction 1) into NEB5A cells. We also transformed backbone 16 into the remaining small amount of cells, thus we had 5 transformations in total.

For each of these 5 transformations, we plated 50ul, then 500ul (the res of the culture) onto the warmed and dried CAM plates. We left the 500ul plates with the lid up rather than the usual agar up, because it was very wet.

The digests had managed to be incubated for around half an hour last night, and were put in the fridge. Tomorrow morning, we will put them in the 37 incubator for another hour and a half or so to let it continue digesting, before running them on a gel.

We also made more PCR product of the sfGFP and the fmT, to have more stock to use for the front and back inserts that are still missing. This was set to run overnight.

13th September:

Took the digests from yesterday out of the fridge and put them in the incubator (around 10.50am) to keep digesting for around an hour and a half. We ran them on 3 gels to see which had digested properly. Based on the results, we concluded that XbaI had inconsistent activity, seeing as it did cut properly in some cases but not in all. We couldn't identify simply from these experiments, what factors were causing the XbaI to malfunction. Based on these digest results, we found that there was one psB1C3 that was fortunately cut correctly by Xba and PstI. We took this sample and PCR purified.

Checked the plates that were spread yesterday, and found that all had grown, but almost all the colonies were too small, so we will let them grow up some more by the end of the day. Picked single colonies from these plates (4 colonies from each plate, and these placed into a single plate divided into quarters to make the single colony stocks), and set up liquid cultures for minprep tomorrow. We only picked from the 1ul ligation from batch 2 - linear (50ul spread), 3ul ligation from batch 1 - plasmid (50ul), and backbone 16 (50ul). We also helped Xenia pick her colonies (4 colonies picked here too).

We took the sfGFP and fmt tubes (4 each) from the PCR and ran these samples on the gel. Pooled them all (as they were all good) and DpnI treated for 1 hour. These were PCR purified afterwards and are now ready to digest as needed.

Checked the sequences for CBDs came out as expected. Confirmed that CBD10 and CBD15 were good, but CBD2 had a mutated base that caused a stop codon instead of a Serine, thus we will send one of the other 5 samples in for sequencing to confirm the CBD2.

Labelled all the CBDs with concentrations and an asterisk for the sequence confirmed tubes.

14th September:

Miniprepped the T7 promotor and backbone 16 liquid cultures, as well as Xenia's cultures.

Nanodropped all these minipreps, as well as the psB1C3 cut with X+P, sfGFP and fmt (2nd batch PCR) that were PCR purified yesterday. The nanodrops were all very high.

15th September:

Finished meeting notes with phil townsend.

Send 3 more samples of CBD2s to sequence (because of the accidental stop codon that was found). Also, sending 3 of the T7 promotor vector that was miniprepped yesterday.

Digest sfGFP and fmT to make stocks for FRONT insert (XbaI and AgeI cuts) and BACK inserts (NgoMIV and SpeI). 50ul digests

Digest all the CBDs for FRONT vector (AgeI and SpeI) and BACK vector (XbaI and NgoMIV cuts) 50ul digests.

Digest these all for about 1.5 hours.

dephosphorylate vectors (3, 10, 11, 12, 13, 14, 15, 16, 17) using following recipe:
25ul vol of digested DNA
3ul rAPid buffer
1.5ul rAPid enzyme
0.5ul dH20

PCR purify all the digests.
Nanodrop them, and calculate the ligations for tomorrow (see details under tomorrow's heading).

Ran about 10ul of the sfGFP+BB and fmt+BB ligation on the gel just to see that the plasmid size is correct. The band sizes seem off, and so we concluded that the ligation didn't work, particularly because the sfGFP and fmt fragments cannot be digested with PstI, which is what we did (cut with X + P). Thus, we can't transform the sfGFP+BB and fMT+BB ligations tonight. :(

Transform instead the CBD clos and cex (becuase we are running out of the high concentration minipreps that we made earlier on), and 1 with RBS and promoter (Amp resisitance) from the 2007 kit distribution plate 3 into DH10B.
Also going to transform the 3 parts from the distribution plates for Laura. (all CAM antibiotic).

16th September:

Ligate the following as 20ul reactions in the morning:
2ul of ligase buffer and
1ul of ligase in all.

1. sfGFP FI + CBD2 BV
   1ul sfGFP FI,
   2.7ul CBD2 BV
   13.3ul dH20
2. sfGFP FI + CBD10 BV
   1ul sfGFP FI,
   2ul CBD10 BV
   14ul dH20
3. NiBP FI + CBD2 BV
   1ul NiBP FI,
   2.7ul CBD2 BV
   13.3ul dH20
4. NiBP FI + CBD10 BV
   1ul NiBP FI,
   2ul CBD10 BV
   14ul dH20
5. fmT FI + CBD2 BV
   0.5ul fmT FI,
   2.7ul CBD2 BV
   13.8ul dH20
6. fmT FI + CBD4(clos) BV
   0.5ul fmT FI,
   5.2ul CBD4 BV
   11.3ul dH20

7. fmT FI + CBD7(cex) BV
   0.5ul fmT FI,
   2.4ul CBD7 BV
   14.1ul dH20
8. fmT FI + CBD10 BV
   0.5ul fmT FI,
   2ul CBD10 BV
   14.5ul dH20
9. CBD4 FV + sfGFP BI
   4ul CBD4 FV,
   1.5ul sfGFP BI
   11.5ul dH20
10. CBD7 FV + sfGFP BI
   9ul CBD7 FV
   1.5ul sfGFP BI
   6.5ul dH20
11. CBD10 FV + sfGFP BI
   3ul CBD10 FV
   1.5ul sfGFP BI
   12.5ul dH20
12. CBD15 FV + sfGFP BI
   3ul CBD15 FV
   1.5ul sfGFP BI
   12.5ul dH20
13. CBD4 FV + NiBP BI
   4ul CBD4 FV,
   1.5ul NiBP BI
   11.5ul dH20
14. CBD7 FV + NiBP BI
   9ul CBD7 FV
   1.5ul NiBP BI
   6.5ul dH20
15. CBD10 FV + NiBP BI
   3ul CBD10 FV
   1.5ul NiBP BI
   12.5ul dH20
16. CBD15 FV + NiBP BI
   3ul CBD15 FV

1.5ul NiBP BI
12.5ul dH20

17. CBD4 FV + fmT BI
    4ul CBD4 FV,
    0.7ul fmT BI
    12.3ul dH20
18. CBD7 FV + fmT BI
    9ul CBD7 FV
    0.7ul fmT BI
    7.3ul dH20
19. CBD10 FV + fmT BI
    3ul CBD10 FV
    0.7ul fmT BI
    13.3ul dH20
20. CBD15 FV + fmT BI
    3ul CBD15 FV
    0.7ul fmT BI
    13.3ul dH20
21. sfGFP FI + psB1C3 backbone
    1ul sfGFP FI,
    3ul psB1C3 backbone
    13ul dH20
22. fmt FI + psB1C3 backbone
    0.5ul fmT FI,
    3ul psB1C3 backbone
    13.5ul dH20

Negative control
(10ul reaction, Ben explained that it's best to use 10ul ligation reactions in future, so as not to waste the enzyme, and since we can only put in about 2-3ul of it in transformations):

3ul psB1C3
0.5ul ligase
1ul ligase buffer
5.5ul water

and

2ul CBD10 BV

0.5ul ligase
1ul ligase buffer
6.5ul water

Got gloves, plates and eppies from stores.

Sent the t7 promotor vector (3 samples) for sequencing with the verification primers.

Autoclaved some LB agar to use for transformation tonight. were poured around 5.15pm.

Need to make a new negative control for the ligations. (the psb1C3 backbone and the CBD10 BV). These backbones are phosphorylated and should not religate or grow on antibiotic plates when transformed.

Set up digests for the potential T7s (tubes 2, 3 and 5) and the confirmed fusions (sfGFP+CBDclos, sfGFP+CBDcex, NiBP+CBDclos and NiBP+CBDcex). I also did an extra digestion using the old XbaI. The others were done with new XbaI that arrived today. We will run them on a gel to see the difference.

These need to be dephosphorylated (the T7 vectors) for half an hour, and then all digests PCR purified for use in ligations tomorrow!

Transform some of the ligation mix into DH10Bs today.
Use one tube of NEB5A for the sfGFP and Fmt in BB.
Plate these on the CAP plates that were made tonight.

Miniprep cultures of the CBD clos and CBD cex were set up by Laura: (2 colonies picked from each 250ul plate). The 300ul plates were stored in the fridge.

The J23038 biobrick extracted from 2007 distribution plate did not grow, and so Ben suggested that we design a primer as soon as possible to use Measkit sfGFP with promotor and RBS from the interlab study as a template, to make a vector with the constitutive anderson promotor J23101 and an RBS.

Dephosphorylate the digested T7 vectors (no. 1, 2 and 3, all digestions are still in the 37 incubator) using following recipe:

10ul vol of digested DNA
2.5ul rAPid buffer

1.5ul rAPid enzyme
1ul dH20

Heat kill the dephosphorylation reaction.

PCR purify all the digests (they are in the red rack in the fridge), ready for ligations tomorrow.

Spread the transformation outgrowths onto CAP plates. As agreed earlier over the phone, we'll spread 200ul and then pellet and freeze the rest of the outgrowth. They have been in the 37 degree incubator room since 7pm. There should be 26 tubes (2 are NEB5A, one or 'sfgfp in bb' and the other 'fmt in bb'), and the transformation tubes are numbered according to the ligations that are on the purple rack in the freezer, but we can stick with the numbers when labelling on the plates until after we miniprep (if we get colonies!).

<u>17th September 2014:</u>
Miniprep biobrick 11 and 12.

Nanodrop the PCR purified digestions (t7 vector and fusion inserts)

Pick colonies from the transformations last night and make miniprep cultures.

Checked sequences for T7 promotor and decide which tube to use for ligations.
Found that tube 2 was the full plasmid with the sfGFP still linked to the T7 promotor.
Tube 3 had a terrible read that didn't match at all with the T7 part we were trying to isolate.
Tube 5 was good, but had a 2 A deletion.
Based on these results and the fact that the T7 promotor is holding us back, we will try to send 3 more for sequencing out of the remaining 5 tubes. We will digest all the 5 remaining tubes based on how they will be used as a vector and run these digests on a gel. and use the ones that look okay for ligations.

<u>18th September:</u>

Get this part from the 2014 distribution:
http://parts.igem.org/partsdb/get_part.cgi?part=BBa_J04500
19J - 2014 Kits Plate 3

Checked the sequences of the T7s sent yesterday. Only tube 6 showed the perfect sequence.

Set up ligations between the successful digested T7s and the fusions that have been digested already (4 tubes of each ligation and 1 for neg control).

Miniprep all of the picked colonies from the 24 transformations.

Prepared water, and cutsmart buffer for the 96x of 10ul digestions
added 3.6 water, and 1ul cutsmart buffer

OR 2.6 water, 1ul NEB 3.1 buffer and 1ul of BSA for MLuI and EcoRV cuts.

Add 0.2ul of enzyme each, 0.2ul enzyme. 5ul of DNA tomorrow.

<u>19th September</u>

Test digest them! (figure out which enzymes to use)

1. sfGFP FI + CBD2 BV MluI and EcoRV (1243, 1926) YES, 1B, 1C* and 1D (faint)
2. sfGFP FI + CBD10 BV MluI and EcoRV (1243, 2271) REPEAT (failed at 2nd attempt)
3. NiBP FI + CBD2 BV NcoI (2 cuts) (842, 1792) YES, 3A*, 3B, 3D (but NiBP BI religation?)
4. NiBP FI + CBD10 BV NcoI (2 cuts) (842, 2137) REPEAT (failed at 2nd attempt)
5. fmT FI + CBD2 BV ApaLI (2 cuts) (1616, 1040) YES, ALL, (with random stuff) 5B*
6. fmT FI + CBD4(clos) BV ApaLI (2 cuts) (1616, 974) YES, ALL, (with random stuff) 6A*
7. fmT FI + CBD7(cex) BV ApaLI (2 cuts) (1001, 1616) YES, 7A* and 7C (with random stuff)
8. fmT FI + CBD10 BV ApaLI (2 cuts) (1385, 1616) YES, 8A* and 8C (but very faint!)
9. CBD4(clos) FV + sfGFP BI MluI and EcoRV (1549, 1554) REPEAT then YES, 9B* worked
10. CBD7(cex) FV + sfGFP BI MluI and EcoRV (1576, 1554) REPEAT then YES, ALL worked, 10D*
11. CBD10 FV + sfGFP BI MluI and EcoRV (1960, 1554) YES, 11D* and 11C (random stuff).
12. CBD15 FV + sfGFP BI MluI and EcoRV (1657, 1554) YES, 12A, 12C*, 12D (but very faint!)
13. CBD4 FV + NiBP BI NcoI (2 cuts) (1420, 1148) REPEAT (failed at 2nd attempt)
14. CBD7 FV + NiBP BI NcoI (2 cuts) (1420, 1175) REPEAT (failed at 2nd attempt)
15. CBD10 FV + NiBP BI NcoI (2 cuts) (1420, 1559) YES, 15B* and 15C
16. CBD15 FV + NiBP BI NcoI (2 cuts) (1420, 1256) YES, 16A*, 16B and 16D

17. CBD4(clos) FV + fmT BI ApaLI (2 cuts) (668, 1922) YES, 17B and 17D* (random stuff still)
18. CBD7(cex) FV + fmT BI ApaLI (2 cuts) (668, 1949) REPEAT
19. CBD10 FV + fmT BI ApaLI (2 cuts) (668, 2333) YES, 19D* (random stuff)
20. CBD15 FV + fmT BI ApaLI (2 cuts) (668, 2030) YES, 20A*, perhaps 20B (random stuff)
21. sfGFP FI + psB1C3 backbone MluI and EcoRV (1243, 1554) YES, ALL, 21B*
22. fmt FI + psB1C3 backbone ApaLI (2 cuts) (668, 1616) YES, 22B*, 22C, 22D (random stuff)
23. sfGFP FI + psB1C3 backbone MluI and EcoRV (1243, 1554) YES, ALL, 23D*
24. fmt FI + psB1C3 backbone ApaLI (2 cuts) (668, 1616) YES, only 24C*.

ApaLI - 8.5 ul minimum
MluI - 5ul minimum.

Run gel with all the test digests! Pick the correct clones from the gel pattern and send them from sequencing and nanodrop them as well. We can throw the non correct ones away, and if there are any that are not correct out of 4, we can pick more colonies.

Transform the ligations and the part extracted from the registry.

Inverse PCR of CBD10 to turn it into CBD 11 and CBD12

4 repeats of GC and no DMSO for turning into CBD10 into CBD11, and another 4 repeat tubes for turning CBD10 into CBD12.
2 negative controls using the different primers but no template added.

Concentration of confirmed CBD10 was 364.9ng/ul

make 100 time dilution: 3.64ng/ul
then use 1ul of this for each reaction tube.

<u>50ul PCR reaction in each tubes:</u>
10ul of 5x GC buffer
2ul dNTPs
2.5ul forward primer
2.5ul reverse primer
1ul CBD10 template (diluted 100 times)
0.5 Phusion polymerase
31.5ul dH20

Mastermix (x10) of:
100ul of 5x GC buffer
20ul dNTPs
315ul dH20

Divide Mastermix into 2, 217.5ng/ul each tube (for the 2 different sets of primers, each x5):
12.5ul appropriate forward primer
12.5ul appropriate reverse primer

242.5 / 5 = 48.5

Then pipette into each of the 10 tubes (48.5), make sure you know which set of primers!

Into the 8 pcr reactions, add the 1ul template

Add 0.5ul of Phusion enzyme into all the tubes.

<u>20th September:</u>
Repeat the digests and gels for 2, 4, 9, 10, 13, 14, 18.
28 digests, 4 gels exactly! (using big wells).

Image the gels.

PCR purified the PCR to make CBD11 and CBD12

<u>21st September:</u>
Inoculate miniprep cultures with the T7 NiBP CBD4, T7 NiBP CBD7, T7 sfGFP CBD4, T7 sfGFP CBD7 and BBa_J04500.
And made stock plates for these.

<u>22nd September:</u>

Digest the confirmed fusions as Back inserts, and run some on the gel.

Prepare sequencing tubes on Saturday for Monday.

If these results are not good, we can pick 5 more colonies from the plates.

We need to digest the “starred” confirmed fusions for BI behind T7.

miniprepped the t7-fusion samples that were picked yesterday, as well as the bba j04500

digested 5ul of this DNA in a 10ul digestion. thus the recipe:

1ul cutsmart buffer
4.2 water
4ul DNA from miniprep
0.4 EcoRI
0.4 PstI

Digesting also the back inserts with XbaI and PstI:
1c
3a
9b
10D – need to nanodrop
11D
11D
12C
15B
16A
21B sfGFP in backbone.
23D

15ul digestion:
1.5ul cutsmart
4.7ul water
3ul of each miniprepped DNA
0.4 of XbaI
0.4 of PstI

prepare tubes for sequencing at 3pm:
1c
3a
9b
10D – need to nanodrop
11D
12C
15B
16A
21B sfGFP in backbone.
23D

Bands expected for:

1) T7 sfGFP + CBD4(clos) - 1109/1125, 2029
2) T7 sfGFP + CBD7(cex) - 1136/1152 2029
3) T7 NiBP + CBD4(clos) - 574/590, 2029
4) T7 NiBP + CBD7(cex) - 601/617, 2029
5) BBa J004500. - 220, 2029

Note that:
T7 about 23 bp
RBS B0034 about 12bp

digest the BBa J04500

Ligations:

FOR ALL:
3ul of J04500 or T7 vector
1ul of T4 ligase buffer
0.5ul of T4 ligase

sfGFP+CBDclos:
5ul DNA
0.5ul water

sfGFP+CBDcex:
4ul DNA
1.5ul water

NiBP+CBDclos:
2ul DNA
3.5ul water

NiBP+CBDcex:
3ul DNA
2.5ul water

1C:
3ul DNA
2.5ul water

3A:
1.5ul DNA
4ul water

9B:
2.5ul DNA
3ul water

11D:
3ul DNA
2.5ul water

12C:

2.5ul DNA
3ul water

15B:
2.5ul DNA
3ul water

16A:
1.5ul DNA
4ul water

24th September:
pick 4 colonies (take 2 glowing out of the 4 if there are) from the J04500 fusions into 8ml miniprep cultures.
Pick CBD11 and CBD12

Miniprep the failed fusions (bad test digests).
Test digest them with Eco and PstI:
3ul DNA
0.2ul EcoRI
0.2ul PstI
1ul Cutsmart
5.6ul water

[the wells looked poor for gel 4 (which had the 4E, 4F, 13E, 13F, 14E, 14F)]
and prepare the good ones for sequencing tomorrow

Restreak the T7 BL21 plates.

Relabelled our tubes and moved our confirmed parts into boxes!

25th September:

Send the 1, 2, 10, that were confirmed by the test digest.
Decided to send: 1E, 2F, 10E.

Miniprepped the samples picked (from the J04500 fusions).

test digest and run on gel. Only a few were successful, and the ones with the bands weren't necessarily the glowing ones. The highly glowing ones had no bands successful!

repeat the test digest for 4, 13, and 14. No successful results from this gel.

Transform T7 NiBP+fusions into BL21:
Ligation no. 12, 13, 17, 18.
plus T7 NiBP + CBD7 and T7 NiBP + CBD4

Found out that some from the plate colony are now glowing (they weren't previously when we picked them) and some of these had good bands during the test gel). Some of the glowing will be sent for sequencing.

26th September:

Sent for sequencing at 3pm:
J1A
J1d
J2c
J6A
J6D
J7A
J8A
J8B
1e
2f
10e
12c
14a

50x 2ml aliquots for IPTG testing. 2 are negative controls using CBD11 which does NOT have a promotor. We made 2 tubes for each colony tested (one which will be IPTG induced after 3 hours and one which will not be!)

Picked colonies for miniprep from the restreak of the T7s in BL21.
and more from the failed ones from the test digest (eg. E, F, G, H for the sfGFP ones with no bands

Made restreaks for the transformations in BL21 from last night (these are mostly T7 NiBP fusions) as these were lawny again this morning.

Blend cellulose and dry on the 96 well plates before this evening!

PCR to turn CBD2 into CBD1.

Get holepuncher tomorrow.

1st October:

We made a new spreadsheet with all potential parts we want to make. By checking through our records, we managed to identify the clones that have not been made or in progress at the moment.

The ones that need more picking because we are low on stocks are:
T7 sfGFP + CBD4
T7 sfGFP + CBD7
T7 NiBP + CBD4
T7 NiBP + CBD7
Biobrick 4

The ones that need to be picked because the sequences came back wrong for the picked colonies so far are:
NiBP + CBD4 in J04500 (This will be 9F, 9G, 9H, 9I, 9J, 9K).
NiBP + CBD7 in J04500 (This will be 10F, 10G, 10H, 10J, 10K).

The ones that are picked from transformations 2 days ago in DH10Bs are:
T7 CBD4+sfGFP
T7 CBD10+sfGFP
T7 CBD15+sfGFP
T7 NiBP+CBD2
T7 CBD10+NiBP
T7 CBD15+NiBP

The ones that need to be ligated overnight today are:
T7 CBD7+sfGFP
CBD7+sfGFP in J04500

T7 sfGFP+CBD10
sfGFP+CBD10 in J04500

sfGFP+CBD2 in J04500

T7 sfGFP
sfGFP in J04500

T7 NiBP

NiBP in J04500
Smt+CBD2
Smt+CBD4
Smt+CBD7
Smt+CBD10

CBD4+Smt
CBD7+Smt
CBD10+Smt
CBD15+Smt

[4 ligations, each need 12ng
48ng each total for front insert (X+A cut) and for back insert (Ng+S cut).]

Also:
Smt into T7 vector
Smt into J04500 vector
Smt in psB1C3

[2 ligations, so only 24ng needed for insertion (X+P cuts)]

Digest in 15ul:
12ul of SmtA
1.5ul cutsmart
0.4ul of XbaI or NgoMIV
0.4ul of AgeI or SpeI or PstI
0.7ul water

T7 vector (set up 3 tubes of this):
S+P

J04500 (set up 3 tubes of this):
S+P

psB1C3 (set up 1 tube of this):
X+P

2nd October:

We will miniprep the picked colonies this morning.
We managed to test digest 24 of the miniprepped samples from 7 to 12 and chose candidates for sequencing.
Sent in today were 7C (T7 NiBP+CBD2), 8C (T7 CBD4+sfGFP), 9A (T7 CBD10+sfGFP), 10C (CBD15+sfGFP T7), 11A (T7 CBD10+NiBP), 12B (T7 CBD15+NiBP).
We also sent the miniprepped J5E (J04500 CBD10+NiBP), J5G (J04500 CBD10+NiBP) and J4F (J04500 CBD15+sfGFP) to sequencing.
The other 24, we didn't digest in time, so we will send them tomorrow.
The correct digests from that batch will be 1B (T7 sfGFP+CBD4), 2B (T7 sfGFP+CBD7), 3A (T7 NiBP+CBD4), 4A (T7 NiBP+CBD7), 5H (J04500 NiBP+CBD4), 6J (J04500 NiBP+CBD7).

3rd October:
As mentioned, will send the following for sequencing:

1B (T7 sfGFP+CBD4),
2B (T7 sfGFP+CBD7), ←--- NEED TO PICK AND MINIPREP MORE, as we sent all to sequence (didn't elute in very much volume).
3A (T7 NiBP+CBD4),
4A (T7 NiBP+CBD7),
5H (J04500 NiBP+CBD4),
6J (J04500 NiBP+CBD7).

Also need to pick more of CBD4 and CBD7 from registry.

We planned the 36 ligations, and divided it into 3 such that it would be 12 ligations for each of us. We labelled it as X1, X2, X3 etc, G1, G2, G3 etc and C1, C2, C3 etc, based on who was looking after that set.

Chris had the gaps in our own cloning so far (this can be seen below). Xenia had the fmt ligations with 1 extra. Gabi had the Smt ligations with 1 extra.

10ul ligation calculations, to all add:
1ul ligase buffer
0.5ul T4 ligase

C1: T7 CBD7+sfGFP:
2.2ul T7 vector

3.2ul CBD7+sfGFP
3.1ul dH20

C2: CBD7+sfGFP in J04500
2.4ul J4500 vector
3.2ul CBD7+sfGFP
2.9ul dH20

C3: T7 sfGFP+CBD10
2.2ul T7 vector
4.5ul sfGFP+CBD10
0.8ul dH20

C4: sfGFP+CBD10 in J04500
2.4ul J04500 vector
4.5ul sfGFP+CBD10
0.6ul dH20

C5: T7 sfGFP
2.2ul T7 vector
2.6ul sfGFP ins.
3.7ul dH20

C6: sfGFP in J04500
2.4ul J04500 vector
2.6ul sfGFP ins.
3.5ul dH20

C7: T7 NiBP
2.2ul T7 vector
0.8ul NiBP ins.
5.5ul dH20

C8: CBD4 + NiBP
1.5 NiBP BI
1.2ul CBD4 FV
5.8ul dH20

C9: CBD7 + NiBP

1.5 NiBP BI
2ul CBD7 FV (Xenia's new stock)
5ul dH20

C10: CBD7 + phytochelatin
4.5 PC BI
2ul CBD7 FV (Xenia's new stock)
2ul dH20

C11: NiBP + CBD10
1.5ul NiBP FI
2ul CBD10 BV
5.0ul dH20

C12: PC + CBD10
2ul PC FI
2ul CBD10 BV
4.5ul dH20

11 ligations with SpeI and 1 extra

1. XbaI FI + CBD2 BV
   1.2ul XbaI FI,
   2.7ul CBD2 BV
   4.6ul dH20

2. XbaI FI + CBD4(clos) BV
   1.2ul XbaI FI,
   5.2ul CBD4 BV
   2.1ul dH20

3. XbaI FI + CBD7(xxx) BV
   1.2ul XbaI FI,
   2.4ul CBD7 BV
   4.9ul dH20

4. XbaI FI + CBD10 BV
   1.2ul XbaI FI,
   2ul CBD10 BV
   5.3ul dH20

5. CBD4 FV + XbaI BI
   4ul CBD4 FV,
   1.2ul XbaI BI
   3.3ul dH20

6. CBD7 FV + XbaI BI
   2ul CBD7 FV (xenia's new stock)
   1.2ul XbaI BI
   5.3ul dH20

7. CBD10 FV + XbaI BI
   3ul CBD10 FV
   1.2ul XbaI BI
   4.3ul dH20

8. CBD15 FV + XbaI BI
   3ul CBD15 FV
   1.2ul XbaI BI
   4.3ul dH20

9. XbaI ins + psB1C3 backbone
   1.2ul XbaI ins
   1.9ul psB1C3 backbone (X+P)
   5.4ul dH20

10. SpeI ins. into J04500 vector
    1.2ul SpeI ins.
    2.4ul J04500 vector
    4.9 dH20

11. XbaI ins into T7 vector
    1.2ul SpeI ins
    2.2ul T7 vector
    5.1ul dH20

12. PC into J04500.
    2ul PC ins.
    2.4ul J04500 vector
    4.1ul dH20

11 ligations with EcoI and 1 extra

1. EcoI FI + CBD2 BV
   4ul EcoI FI,
   2.7ul CBD2 BV
   1.8ul dH20

2. EcoI FI + CBD4(clos) BV
   4ul EcoI FI,
   5.2ul CBD4 BV
   No dH20

3. EcoI FI + CBD7(pex) BV
   4ul EcoI FI,
   2.4ul CBD7 BV
   2.1ul dH20

4. EcoI FI + CBD10 BV
   4ul EcoI FI,
   2ul CBD10 BV
   2.5ul dH20

5. CBD4 FV + EcoI BI
   1.2ul CBD4 FV,
   4ul EcoI BI
   3.3ul dH20

6. CBD7 FV + EcoI BI
   2ul CBD7 FV (xenia's cut stock)
   3.5ul EcoI BI
   No dH20

7. CBD10 FV + EcoI BI
   3ul CBD10 FV
   4ul EcoI BI
   1.5ul dH20

8. CBD15 FV + EcoI BI
   3ul CBD15 FV
   4ul EcoI BI
   1.5ul dH20

9. EcoI ins. + psB1C3 backbone
   4ul EcoI ins.
   1.9ul psB1C3 backbone
   2.6ul dH20

10. EcoI ins. into J04500 vector
    4ul EcoI ins.
    2.4ul J04500 vector
    2.1 dH20

11. EcoI ins into T7 vector
    4ul EcoI ins.
    2.2ul T7 vector
    2.3ul dH20

12. NiRR in J04500
    2.4ul J04500 vector
    0.8ul NiRR ins.
    5.3ul dH20

Checking sequences that came back:
7C (T7 NiBP+CBD2) → CONFIRMED
8C (T7 CBD4+sfGFP) → does not have T7 part
9A (T7 CBD10+sfGFP), → does not have T7 part
10C (CBD15+sfGFP T7), → does not have the T7 part :(
11A (T7 CBD10+NiBP), → does not have the T7 part :(
12B (T7 CBD15+NiBP) → does not have the T7 part :(

Based on the above results, we concluded that we need to find a new way to determine whether the T7 part is included in the clone. We decided to do the same test digest, or a digest with a cut in the prefix and one in the insert, such that the 30bp difference of the T7 can be read on a 2% gel. We would also be running the normal fusion next to the potential digested clones in order to see the 30 bp difference.
Michael also suggested a 'culture PCR' which is a variation of a colony PCR, but using miniprep liquid cultures. By using the biobrick verification primers, we'd be amplifying the length of DNA that would usually be seen through test digest but using green GoTaq. The pcr product can then be run on the gel and easily imaged. This is ideal as an alternative to miniprepping and test digesting, since you can inoculate the liquid cultures directly from the transformation plates. We may use this method for higher throughput!

We transformed the ligations we had set up earlier in the morning. We transformed most of them into NEB5A because we had run out of the chemically competent DH10Bs. Some of them were in DH10Bs though, these were all of the Gs and the C1.

We spread these and let them grow overnight.

4th October:
We checked the plates and the DH10Bs had grown but most of the NEB5A were looking quite small or didn't grow. We will let them grow for longer in the incubator and see if we can pick some later tonight.

We need more competent cells, both BL21 (to express our sequenced confirmed T7s and J0s) and DH10Bs (to move our fusions into the T7 vector). Thus, we inoculated 2ml of LB with some competent BL21s and DH10Bs. We let this grow in the 15ml clip top tubes in the 37 shaking incubator for 1 hour, then used all of this starter culture to inoculate 125ml of LB each. This went in the incubator in a conical flask with a foam bung, and cultured for 3 hours.

With the remaining DH10Bs in the same tube, we transformed the two that needed transforming (T7 sfGFP+CBD2 (lig. tube 12 - 1C+T7) and J04500 NiBP+CBD2(lig. tube 6 - 3A+J0)). These ligations were done on the 23rd of September. We had forgotten about transforming these two yesterday because of the rush.

We also set up these inoculations in the incubator to do a preliminary assay on a 96 well plate:
1a – sfGFP+CBD2 in J04500
2a – CBD4+sfGFP in J04500
3a – CBD10+sfGFP in J04500
7a – sfGFP+CBD4 in J04500
8b – sfGFP+CBD7 in J04500
6a (NiBP fusion in J0) – CBD15+NiBP in J04500
Measkit GFP/strong promotor.
CBD11 in psB1C3

These cell lysates will be arranged along the rows.

We will try 4 different washes:
Water
Ethanol
PBS (with Tween)
BSA (with Tween)

and these 4 washes will be done in triplicates across the columns of the plates. (eg. column 1-3 will be washed with water, column 4-6 will be washed with ethanol etc.)

We also prepared a plate with 200ul of thick blended cellulose (this choice was made by previous trials) in each of the 96 wells flat bottomed wells. This was left in the oven 60 degrees to dry. We will be using this plate during the preliminary assay.

We pelleted the inoculated cultures, and resuspended this in PBS. These cell resuspensions were sonicated to lyse them, and release the cell lysates to use for the preliminary assay.

We applied the cell lysates to the appropriate row on the cellulose column and left it to adsorb for 12 hours at 4 degrees celsius in the fridge.

5th October:

We read the cellulose plate and had results as we expected and hoped for (ie. the GFP alone washed off after the 3 washings of water, ethanol, PBS, and BSA; whilst the sfGFP-CBD fusions had more lasting power.

The readings were taken pre-wash and post wash to show decrease in fluorescence. The remaining fluorescence can be assumed to be attached via CBD onto the cellulose. Percentage difference for each cell lysate pre-wash and post wash will account for differences in protein concentration between the different cell lysates.

These results will be replicated on a larger scale with more controls to generate comparable data to form graphs and as part of our characterisation.

We also picked colonies from the 36 transformations. We will be colony PCRing 5 colonies from each plate, with hopes that at least one from each transformation will come up positive on the gel. We're hoping to use the Biobrick verification primers to PCR them out, and for the T7 transformations, we will be doing a further PCR with the T7 reverse primer and the forward verification primer in order to ensure that there is a T7 promotor part included. This will avoid the issue with our test digested that we had previously when we sent sequences in without the T7, even though they passed the test digest.

-Set up PCR to turn CBD2 into CBD1
-Set up PCR to turn CBD11 into 13
-Set up colony PCRs before we miniprep.
-Run on gel when we get more ladder.
-Fill in gaps for T7 by picking more colonies and colony pcr-ing.
-Retransform CBD4+Smt, and Smt in J04500
-Pick colonies from the 2 DH10B transformations that we put into incubator in the morning.

-Send the email to Kirsten with the different permutations of our fusion that we want to purify. Check that she has ordered the cellulose beads, and also the status for SDMing the CBD10

-Transform into BL21s the confirmed T7 fusions and induce with IPTG.

Saturday 11th October:

Meet Kieran in the morning to ask about the different fusion proteins and how to purify them.
Email Kirsten with the list of proteins we have and want to purify, and also
DpnI.

Sunday 12th October:

PCR purify (with zymo kit) the CBD10 SDMs:

1. CBD10 +psb1C3
2. CBD10+NiBP
3. sfGFP+CBD10
4. CBD10+sfGFP
5. C3E
6. J04500 CBD10+sfGFP
7. CBD10+PC
8. CBD2+psB1C3
9. C3c
10. C11D
11. C12A
12. G4E
13. G7A
14. X4B
15. CBD12+psB1C3
16. CBD11+psb1C3
17. C3D
18. C11A
19. X7E
20. X7D
21. PC10 I
22. 15C
23. 15B
24. 31A
25. 31B
26. 17A
27. 17B
28. 33A
29. 33B
30. CBD10+sfGFP, Back inserts to turn into CBD12 and then needs SDMing.

31. J04500 CBD10 +sfGFP, Back inserts to turn into CBD12 and then needs SDMing.
32. Turning CBD2 into CBD1
33. CBD13 in psB1C3

Ligation mix (15ul)
DNA elution 10ul
2ul water.
1ul buffer
0.5ul ligase

Blunt end ligate on the bench top for 1.5 hours.
Heat kill ligation mix.
Transform into DH10Bs and BL21 as appropriate.

Pick colonies tonight (make plate stock), let grow.
Miniprep and test digest on Monday.
Send on Tuesday.

Remember to tell kirsten that we want to send something on Tuesday.

We also want to transform the PC fusions (J04500 and T7) into DH10Bs and BL21s (Xenia is doing this)

and then we want to sent up cultures with these:

dated 2nd Oct:
J4F – CBD15+sfGFP J04500
dated 4th or 3rd Oct:
1a – sfGFP+CBD2 in J04500
2a – CBD4+sfGFP in J04500
3a – CBD10+sfGFP in J04500
7a – sfGFP+CBD4 in J04500
8b – sfGFP+CBD7 in J04500
6a (NiBP fusion in J0) – CBD15+NiBP in J04500
CBD11 in psB1C3
3 tubes sfGFP RFC25 in J04500!! Pick more colonies and run the colony PCR?

CBD cloning
CBD expression and characterisation