Lab Book

17/07/14

Laboratory Rules:
1. Wash your hands before and after working in wet lab area
2. Spray ethanol on your bench before and after working in wet lab area
3. LABEL THINGS PROPERLY
    → Add date, your initials, and short sample description
4. Take your gloves off before heading over to dry lab area
5. Please keep sterile conditions when working with stocks! Make your own stocks out of the big stocks so that we keep these sterile
6. Change tips! If you are unsure whether you have contaminated your tip, bin it! Better be safe than sorry
7. If you are not sure about how to proceed, ask your lab partners! They may be able to help
8. Keep benches TIDY

To do list:
1. Find and print protocols
2. File these in binder, keep them in order
3. Get tags and thin-tip sharpies for labelling
4. Figure out ways/places where to grow *Acetobacterium* en masse
    -Acetobacterium containers
    -Container lids for temp. control
    -Thermostat/ temperature control
    -Physical place to grow them
5. Contact people from Imperial Polymers and Composite Engineering about Acetobacterium growth - http://www3.imperial.ac.uk/polymersandcompositesengineering/aboutpace

18/07/14

1. Research

2. Preparing media
    1. 3x500ml Acetobacter Liquid Media
    Glucose - 10 g
    Peptone - 2.5 g
    Yeast extract - 2.5 g
    Na2HPO4 - 1.35 g

Citric acid - 0.75 g
Distilled water - 500 ml

2. 500ml Acetobacter Agar Media
Glucose - 10 g
Peptone - 2.5 g
Yeast extract - 2.5 g
Na2HPO4 - 1.35 g
Citric acid - 0.75 g
Distilled water - 500 ml
Agar - 7.5 g

Must be autoclaved
NB! add glucose to 200ml and the rest to 300ml of distilled water; autoclave solutions separately and mix using sterile techinque post autoclaving, to avoid potentially toxic components arising from Maillard reaction (sugar+amino acids at high temp.)

3. Trying out Suzanne Lee's protocol with sucrose (common sugar)
Materials:
- 1x200ml organic cider vinegar
- 200g granulated sugar
- One piece of live Kombucha culture
- 2x tea bag

Procedure:
1. Boil 2L of water to 100degrees
2. Pour into container
3. Add green tea and allow to brew for 15minutes
4. Remove tea bags
5. Add sugar (200g)
6. Stir until dissolved
7. Cool down to below 30degrees
8. Add organic cider vinegar (200ml)
9. Add one piece of live Kombucha culture. It will sink to the bottom of the container
10. Cover growth container with breathable cloth and grow at 25 degrees (oven)
11. Fermentation starts after 48-72h, thin skin and bubbles will be produced and culture will come back out on the surface
12. When product becomes about 2cm thick, take it out
13. Wash with soaped water
14. Let dry

Considerations:
→ To ensure an even surface, dispel the bubbles towards the edges

4. Plating
1) We isolated 2x4ml of liquid culture, 2x7ml of liquid + a small piece of Kombucha culture
Labels are as follows:
18/7/2014 Kombucha sample 1-2 (liquid+piece, aka solid culture)
18/7/2014 Kombucha liquid 1-2 (liquid only)
2) We have stored these samples on the bench at room temperature
3) Prepared plates with NO antibiotic, poured roughly 25ml of GX agar medium
4) Prepared 6 plates, all from solid culture #1 and with NO antibiotic
    2x streaking plates
    1x 10ul
    1x 50ul
    1x 200ul
    1x 500ul
5) Growing overnight at 26-27 degrees

5. Checking on presentation

6. 3pm meeting with the Centre
Considerations from the meeting:
→ Test B.subtilis for protein production (functionalization) and secretion because it is a much better host for protein expression
→ Real time reporting of what contaminants are present in the water, maybe by image analysis or biosensing system to release colours

19/07/14

1. Checking on plates
→ No growth but no contamination

2. Research

21/07/14

1. Checking on plates
→ Growth successful

2. Checking on fermentation trays

→ Film starting to arise

3. Research

4. Inoculating Gluconacetobacter media with single colonies from different plates (4 small and 4 large colonies). 15mL of culture used; sterile technique used throughout.

22/07/14

1. Checking on plates
→ Growth successful

2. MF - Microscopy of growing colonies

- Used 5x and 10x magnification to compare colony morphologies
    - Three distinct morphologies observed: Large round, small round, small irregular. Large and small round likely both yeast
- Dissolved 1 large and 1 small irregular colony in dH20, suspended by pipetting. Added 10ul to a microscope slide and observed under 100x magnification. Large colony sample had distinctly yeast (budding yeast) cells; small irregular colony samples had much smaller, rod-shaped cells, most likely a bacterial species. Hopefully Gluconacetobacter xylinum.
- Inoculated approximately 5ml of Gluconacetobacter medium from the microscope sample; placed in standing incubator at ~28C.

24/07/14

MF - Monitoring growth and microscopy

Checked growth of colonies from re-streaked plates and culture inoculated with G.xylinum on 22nd July. Colonies on re-streaked plates all small, same morphology as previously observed for bacterial colonies – most likely G.xylinum. Prepared 3 slides from culture – very low number of cells present, hint of cellulose fibrils - very few seen under microscope, could be contamination/clumping. Low growth possibly due to improperly prepared media (glucose and amino acids autoclaved together, which may result in toxic compounds due to Maillard reaction).

25/07/14

MF - Monitoring growth and microscopy

Readings: temp. of samples 0721MFI to 0721MFVIII and 0722MFI 30C, not shaking. A thin sheet visible in the flasks of 0721MFI to IV and 0722MFI (inoculated from small colonies), thick cloud in 0721MFV-VIII. Prepared microscopy slides of all cultures. Microscopy suggested that there are no contaminants in cultures 0721MFI, IV and 0722MFII, thus we have hopefully isolated a pure strain of cellulose producing *G.xylinum* or (another species) from Kombucha culture.

26/07/14

MF - Aliquoting *G. xylinum* cultures

- Made 1ml aliquots of cultures 0721MFI (aliquot named 0726MFI), 0721MFIV (aliquot named 0726MFII) and 0722MFI (aliquot named 0726MFIII).
- Plated out 100ul of 0721MFI, 0721MFIV and 0722MFI onto Gluconacetobacter-agar media. Incubated at 30C.
- After plating, discarded all 0721MF and 0722MF cultures.

27/07/14

MF

- **Microscopy of *G.xylinum* colonies**
  - Observed plates of 0726MFI – III under low magnification (50x); no contamination was detected in any of the plates. Colony morphology roughly similar, although 0726MFI colonies appeared often larger than 0726MFII (this could be attributed to small differences in e.g. temperature or aeration).
  - 100ul is too much, as colony density was high – for obtaining single colonies, 10-20ul should suffice.

28/07/14

MF

- **Inoculating *G.xylinum* cultures from single colonies -** A single colony was marked on each of 0726MFI – III plates and used to inoculate 15ml of *Gluconacetobacter* medium

(in a 50ml tube). Cultures were placed on 30C, no shaking for growth. Sterile technique was used throughout.

- **Microscopy of *G.xylinum* colonies -** Observed plates of 0726MFI – III under low magnification (50x); no contamination was detected in any of the plates. Colony morphology roughly similar, although 0726MFI colonies appeared often larger than 0726MFII (this could be attributed to small differences in e.g. temperature or aeration). 100ul is too much, as colony density was high – for obtaining single colonies, 10-20ul should suffice.
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29/07/14

MF - Monitoring culture growth

- Temp 28C, no growth or cellulose observed in any cultures

30/07/14

MF - Monitoring culture growth

- Temp 30C, thin cellulose sheet observed in samples 0728MFII and III, not in 0728MFI.

XSM with DP - Transformations of biobricks into cloning strain DH10B

- Took the plasmid DNA from the Kit plates with Ben:
  Added 10ul dH20 to each of the relevant wells, mixed up and down a few times, took out 3ul in eppendorf. Plasmids were numbered as follows:

1. fMT - K190019, Spring 2011 Plate 3 well 14D, CAM
2. SmtA - K519010, 2014 Plate 1 well 12O, CAM
3. Laccase - K863000, 2014 Plate 2 well 13I, CAM
4. T7-RBS-sfGFP, I746909, 2014 Plate 3 well 8M, CAM
5. pBad-araC-sfGFP, I746908, 2014 Plate 3 well 5C, CAM
6. pBad-araC, I0500, 2013 plate 5, well 14N, CAM

- Also took out the Transformation efficiency kit and the backbone plasmids and put in our freezer
- For the transformations: 50ul chemically competent DH10B aliquoted out from the -80degC stocks. Added 2ul of DNA (no smaller pipette :( ). Heat shocked 42degC 45s.

Ice 2min. Added 300ul LB, incubated 37degC in shaker (insert make) ~40min. Plated out 100ul with Des.

- No. 7, the +ve control, was the 50pg/ul DNA from the transformation efficiency kit.

31/07/14

XSM - checking on transformations and miniprep cultures

- A few small colonies for 1 to 5, the most on 1 then only a few on the others
- No colonies for 6 (KAN plate was 100ug/ul, low salt) or 7 (+ve ctrl!)
- For the miniprep cultures: 5ul 1000x CAM added to 5ml LB in a 50ml Falcon tube. Innoculated with single colony using a pipette tip. Placed in incubator room (not sure of temp, need to check? it was the first door) and grown o/n at 150rpm.

01/08/14

XSM

- Took out the miniprep cultures from the incubator room after they had grown ~15hrs and placed in fridge, lef tthere ~8hrs.
- Minipreps were performed using QIAgen protocol (no optional PB wash step) with CM and DP. DNA eluted in 30ul autoclaved dH20 and stored at -20degC

04/08/2014

XSM

- Streaked out the parts which arrived from the registry as agar plugs/stabs (plus see biobrick document for detailed part info:
  - K1159002 - Laccase from *B.pumilus* - CM
  - K1012001 - RP1 (binds microcystin) - DP
  - K1151001 - NiBP - DP
  - K863101 - CBDcex - CM
  - K863111 - CBDclos - XSM
- Also arrived was K231001 - Phytochelatin synthase, but wasn't streaked out because it needs kanamycin antibiotic which we don't have yet.
- BioBrick K925007 - MyToxic - this one did not arrive.
- The agar plugs were stored at 4degC, the linearised backbone and RFP construct were stored at -20degC.

05/08/2014

HC

- Cut out 4 samples of cellulose, labelled from 1-4, from the first batch of cellulose.
- Made a solution of 1l of 0.1M NaOH.

- Immersed sample 1,2, 3, and 4 in 140,125,100,80 ml of NaOH in beakers with Alu foil covering the top. Inoculated at 120C for 1 hour in oven, NaOH turned yellow.
- Sample 1 was completely white and blended for 5 min at stage 2 to form a homogenous solution. It was poured into a weighing boat and left overnight with cling film covering.
- Sample 2 was washed with water and immersed in a solution of sodium bicarbonate overnight as suggested by Zuzana to remove the smell.
- Sample 3 & 4 were incubated overnight in NaOH at 120C because they had not changed colour entirely during the 1 hour incubation.

XSM

- Mini-prep cultures - 5ml LB plus 5ul CAM, innoculated with single colony:
    - K1159002 - Laccase 2
    - K1012001 - RP1
    - K1151001 - NiBP
    - K863101 - CBDcex
    - K863111 - CBDclos
    - the kanamycin construct was not done as we need the antibiotic

06/08/2014

HC

- Checked on sample 1, about 50% water has evaporated. Upon correspondence with Koonyang, the solution was poured onto VWR 213 filter, and left on top of blue roll.
- Checked on sample 4, completely white but some residual smell from NaOH. It was washed with water and put in a press between two cling film wrapped tiles. Upon correspondence with Koonyang, the sample was moved on to a VWR 213 filter and sandwiched between another one on top. A weight was added on top of 5 tiles, approximately 1.5kg - the glucose container.
- Sample 2 had slightly darker edges perhaps due to too short NaOH incubation, but not residual smell whatsoever. Left in the tile press to dry, to allow comparison between using baking soda treatment and not.
- Sample 3, blended for 4:45 at speed 2. A volume of 52ml was added, as it was found to be the minimum possible for the blender to operate. Left to dry in a weighing boat, which was transferred to a VWR filter upon correspondence with Koonyang the man!
- Prepared two samples of cellulose with Chris M. & Despoina. They were treated with NaOH and left to dry in the oven at 60C. One in a press and one without, both on filter paper.
- Prepared two samples of cellulose for drying at room temperature without NaOH treatment to test Susanne Lee's method of drying. One sample is in a press, the other is air drying. Both on VWR filter paper.

XSM

- Mini-prepped the DNA with DP and CM. Included the PB wash step too. Eluted with 30ul autoclaved dH20

08/08/2014

MF

- Installed an aeration system (based on aquarium air pumps) into 3 trays. Made holes along aeration tube, to evenly distribute it
- Got filtration results. The cone shaped filter produced the highest flow rates of 0.14 ml/min and 0.058 ml/min for two different filters. The flat filter sucked and produced a flow rate of 6 ul/min.

CM

- Pulled out 28 Biobricks from various distributions (numbers 13-39 in our Biobricks spreadsheet). One of the Biobricks seems to be a mystery one which probably is just likely dye.
- Did so by addition of 10ul of dH2O with mixing up and down a few times, then aliquoting 5ul of this into eppendorfs, before freezing the distribution plates again.

09/08/2014

HC

- Checked overnight filtering with non NaOH treated BC in a flat configuration, the flow rate was 3.8 ml/min.
- Resoaked non-treated BC and a NaOH treated BC sample, blended the the samples in the Jamie Oliver blender and found an issue with it getting cellulose stuck in the bottom. Sorted it out. Blended produce looked great, and poured it on 413 filter that has been put to dry in the oven at 45C. Picked out the chunks from blended BC to get a uniform filter, and distributed the lumps with a spreader.
- Checked samples that had been NaOH treated for 20 min and then immersed in dH20 to remove the colour. The colour removal was unsuccessful.
- Transformed 14 parts and made kanamycin plates. Plated all 26 parts transformed.

GS

- The Biobrick parts taken from the distributions were labelled with letters (the key in listed my notebook and in Chris').
- With ChrisM and others, transformed competent DH10B cells with the 27 Biobrick parts (excel no. 13 to 39 on the Biobrick inventory) taken from Registry. There was 1 'mystery' part (repeat of J23101) that will likely just be dye, so there should not be any transformed cells. Remember to note observed growth on that plate.
- Spread the transformed cells onto the appropriate antibiotic plates and incubating at 37 degrees overnight.

MF

- Growth room temp 24-25C, all cultures growing.

11/08/2014

GS, DP, CM and HC

- 'Miniprepped' the 25 transformed cultures (Ben picked the colonies over the weekend and left 2 plates that did not grow (13 and 38 (I think?!).
- We opened a new Miniprep set and forgot to add the RNase to the P1 buffer, but had already accidentally done this to 6 samples (13/C, 28/N, 36/Q, 39/Z, 20/O, 21/W), so we set up new miniprep cultures for these samples. We put them into the shaking table in the 37 degree room.
- Prepared 8 x 800ml of LB agar to make stock agar plates of various antibiotic resistances.
- We 'nanodropped' all the 25 plasmid samples miniprepped today, and the samples that Xenia had done before (10 samples).
- Identified the samples that were below 80ng/ul, and we re-picked these colonies from the plates and re-cultured them in 6ml of LB broth with 6ul of antibiotic. These were put into the 37 degree room to grow overnight. The 11 samples were: 16ø, 18A, 27M, 30L, CBDclos, CBDcex, PP1, fMT, Laccase 2 (K1159002), T7 sfGFP, NiBP. We inoculated the CBDclos with 2 colonies.
- Removed the 6 samples that we recultured earlier in the day, and stored them in the fridge. We will miniprep them tomorrow with the 11 samples that are still in the 37 degree room.

12/08/2014

XSM and GS and CM

- Set up restriction digests of BioBricks which need assembling into devices for the Interlab study. The two different promotors (K823005 and K823012) need cloning in front of the GFP coding device (E0240) or RFP (extra credit, found BBa_E1010 but realised this has no RBS /terminator so will redo with a different part.)
- At first, the upstream parts (17 and 32) were digested with EcoR1 and Spe1, the downstream (14 and 15) were digested with EcoR1 and Xba1. However, realised that this digestion method would result in a very small fragment of ~40bp for the promoters (upstream parts), and when this was ran on a 1.5% agarose gel it was lost. So, in the evening a second digestion was set up for 17, 32 and 14 (not the 15 rfp as this is wrong part) with Spe1 and Pst1 for the forward parts (17,32) and Pst1+ Xba1 for the downstream parts. This way, the backbone plasmid is preserved in the upstream (17, 32) parts rather than the downstream parts.
- Reactions were set up as follows

Construct:	17 (K823005)	32 (K823012)	14 (E0240)	15 (E1010) N.B. not done for the 2nd round

DNA (<1ug advised, calculated 900ng)	6.9ul	9.6ul	9.7ul	8.5ul
Buffer (10x)	2ul	2ul	2ul	2ul
Enz1	0.75ul	0.75ul	0.75ul	0.75ul
Enz2	0.75ul	0.75ul	0.75ul	0.75ul
dH20 to 20ul	9.6ul	6.9ul	6.8ul	8ul

- All Buffers and enzymes are from Invitrogen (insert product numbers here)
- The buffer used was calculated using Invitrogen's compatible buffers for double digest table from http://tools.lifetechnologies.com/content/sfs/brochures/CO13950-Restriction-Enzyme-Chart_LRF.pdf
- Amounts in table above were based on the suggested amounts in the enzymes instruction manual online, and that when doing digest the amount of enzyme should not be more than 10% total rxn volume as they are in glycerol.
- A 1.5% agarose gel (with SYBR Safe Gel stain, Invitrogen) for the first (incorrect) round was run (2ul 10x loading buffer, (Invitrogen) was added to the sample, ladder Invitrogen TrackIt 1kb Plus #10488-085, 120V ~35min) and bands for 14 and 15 cut out and placed in eppendorf tubes in the fridge. The ~40bp fragment was not visible on the gel and was lost.
- The second round digestion for 17,32 and 14 with the alternate enzymes was set up and left at 37degC overnight. N.B. realised another mistake, number 32 is actually J23116 not K823012, I looked at the wrong number. See the fragment descriptions tomorrow.

GS, HC, DP and LG

- Took the cultures that we were planning to miniprep today and instead set up 3 x 7ml (2ml from the pre-culture and 5ml new LB broth with 7ul of antibiotic) cultures per plasmid, in 3 plasmids. There were 18 of the pre-cultures and we've made 54 Falcon tubes of cultures. Tomorrow, we will pellet the triplicates into one pellet, and then miniprep these 18 samples. We prepared labelled eppendorfs for the minipreps.

13/08/2014

XSM

- 1.5% agarose gel ran as described previously (120V, 45min), for the restriction digests that had incubated overnight. Bands were excised and DNA purified (including some from the first round yesterday) using the QIAgen PCR purification kit. N.B. the isopropanol step was left out. Purified DNA concentration was measured using the nanodrop spectrophotometer. Purified fragments are as follows:
    - 14/E0240 cut with E+X=linearised pSB1C3 with E0240 after the X.

- 15/E1010 cut with E+X=linearised pSB1C3 with RFP CDS after the X.
- 17/K823005 cut with S+P = linearised pSB1C3 with J23101 promoter before the P site.
- 32/J23116 cut with S+P=S-RFP coding device-N-P fragment
- 14/E0240 cut with X+P = X-GFP coding device-S-P fragment
- The only two here which are compatible for ligation into the desired product are the cut 17SP and cut 14XP.
- Purified fragments stored at -20degC

CM

- Ran the gel from the overnight digestion with XSM.
- Resuspended the lyphalised primers that had arrived and prepared stocks:
  - Added in ul of dH2O 10x nM conc written on the side of each vial to make master stocks.
  - Then aliquoted 10ul of this for each one and made it up to 100ul with dH2O to make working stocks labelled 1-22 in the primer box in the -20 degrees freezer.

GS, HC and DP

- Miniprepped the 18 samples that were cultures in 3 different falcons. Nanodropped them and all values are above 100ng/ul. Stored these plasmids in the freezer.

MF

- **Transforming *E.coli* BL21(DE3) with biobricks -** Followed chemical transformation protocol from XSM. NB! Water bath temp. might not have been fully at 42C, but lower. This could cause massively decreased transformation efficiencies. NB! It seems the thermometer was broken, so real temp. Plated out on LB-agar with ampicillin, left to grow at 37C.
- **Culturing Kombucha-isolated *G.xylinum* on different media -** Set up 15 shaker flasks, autoclaved them. Measured OD_{600} of Kombucha-isolated *G.xylinum* using Nanodrop – concentration below detection limit, so Acetobacter requires further culturing.
- **Testing natural antibiotic resistance of Kombucha isolated Acetobacter -** Could not continue yet as seed culture was not ready (OD600 very low)
- **Making glycerol stocks of Kombucha-isolated G.xylinum -** Can use seed culture (after digestion with cellulase) when it is ready.
- **Gluconacetobacter 3D growth -** Set up two bioreactors (5L measuring flasks with 3L culture, aeration from below using fish tank aerators) and two trays with BR. Added approximately 15 cotton balls to both bioreactors. For one bioreactor, injected blendered Kombucha culture into the centre of the balls, for the other, simply added to the culture medium. For two trays, added 1.5L of Kombucha tea medium each, and placed newspaper or blotting paper on the surface of the medium to provide a 2D scaffold for G.xylinum.

14/08/2014

MF

- **Transforming BI21(DE3) with Biobricks** - Transformants from yesterday are growing – colonies present on samples 0813MFVI – 0813MFVIII.
  Inoculated two colonies from each plate into 20ml LB+amp medium (in 50ml Falcons) for minipreps. Grew with shaking at 37C, 140rpm (due to problems with higher shaking speed) overnight.
- **Culturing G.xylinum strains on different media -** Set up and autoclaved 15 additional flasks for growing ATCC23769 strain. Waiting for seed culture to grow in order to begin the experiment.
- **Testing antibiotic resistance of G.xylinum strains**
  Set up additional HS-antibiotic containing Falcon tubes (50ml tubes containing 15ml of HS+ ampicillin, chloramphenicol or kanamycin). Waiting seed culture to grow to begin the experiment.

GS, XSM and CM

- Prepared samples to send for sequencing at Sourcebio.
- Ran Touchdown PCR (70 degrees to 55, then 20 cycles at 55 degrees for annealing) with Phusion on the sfGFP to turn it into Freiburg format (primers with 'flappy ends" to add the freiburg sites).

15/08/2014

LAG

- **Genomic DNA extraction out of Acetobacter Xylinum ATCC 23769**
  Initial volume= 10ml 48h acetobacter culture
- **DNA amplification:**

  Amplification of the Acs Operon (AcsABCD)
  Forward primer = 5' TTA CAG CAT TTC GGA CGA GTT G 3' T.m= 63.8
  Reverse Primer = 5' CGT TGT CCT GCC TCA GGT 3' T.m= 64.1
  Simultaneously, we'll amplify the AraC-pBAD-pSB1C3 vector to produce a linearized, blunt vector
  Forward primer = 5' TAC TAG TAG CGG CCG CTG CAG 3' T.m= 69
  Reverse primer = 5' GCT AGC CCA AAA AAA CGG GTA TGG AG 3' T.m = 69.2

  Program:
  98 degrees 30 seconds
  98 degrees 30 seconds
  50-52-54-56-58-60 (insert)/60-62-64-66-68 (plasmid) 15 seconds
  72 degrees 12 minutes x25-30 cycles

72 degrees 10min
4 degrees

CM

- Performed PCR purification of the Freiburg-ed SFGFP PCR product from the previous day as per the QIAGen PCR Purification kit and eluted in 30ul in water - stored at 4 degrees, labelled “Freiburg SFGFP 2.5/5.0/10.0ng PCR 15/08014”.

HC

- **Checked & treated pellicle from initial Kombucha setup.** The pellicle had been treated for 2 days in a distilled water solution. Water had turned brownish and a thin film had developed underneath that was peeled off to reveal a whiter layer of cellulose.
- **Set up samples for treatment**
    - 3 samples baking soda treated, 120C
    - 2 samples dH20 treated at 120C
    - 1 large sample for leather treatment
    - 2 thin filters drying in air, taken from film under pellice. Less than 0.2mm thickness.
    - 2 samples left at -20C overnight, both NaHCO3 treated
    - 2 samples left at -80C overnight, one NaHCO3 treated and one NaOH treated.
- **Sample results.** Both frozen samples displayed no change in structure due to freezing, and rapidly returned to a liquid state upon touch. The distilled water treatment produced better results than that of baking soda, NaHCO3 in terms of whiteness.
- **Send sample to Phil Townsend.** Found a dry sample and send off to Phil.

16/08/2014

LAG

- Gel Electrophoresis → Preparing a .5% Agarose Gel, running at 110V for 30min

    Preparation
    50ml 1x TAE buffer
    0.25 g Agarose
    4ul Sybr Safe
    Sample Loading
    10ul sample
    1.5ul 10x loading dye
    7ul 1kb marker

GS, CM and XSM

- Digested the sfGFP PCR fragment (now in Freiburg format) and a vector backbone. We chose Biobrick number 16 because we have high concentrations of this and it is in the

right plasmid. We have to phosphorylate the linearised vector however we don't have CIAP only Antarctic Phosphatase which will take much longer so we will do this Monday.
- Set up 50ul PCR reactions for fMT, SMTA, NiBP and did more of sfGFP so that we can do some parallel ligations (into the correct backbone for submission into the Registry, with the two CBDs and the laccase that are already in Freiburg format).
    - PCR was carried out using the same "Phusion Touchdown" programme used previously - edited to include an extra 5 cycles at 55 degrees to maximise PCR product (total: 15 cycles with decreasing temperature from 70 to 55 degree then 25 cycles at 55 degrees).

MF

- **Growing up and testing pure ATCC23769 strain sent from U.Röslig lab.** Prepared new HS media to remove possibility of contamination due to contaminated media, inoculated using sterile techniqe.
- **Testing ATCC23769 for contamination -** Plates grown overnight were filled with small colonies (Acetobacter colonies are expected to be visible within 48h, not overnight), with *E.coli* colour and morphology (as viewed under microscope with 50x magnification). Furthermore, plates smelled like *E.coli*, all of which (too fast growth rate, colony morphology and cloudy liquid cultures) points to *E.coli* contamination. Thus, PCR done yesterday (with supposed ATCC23769 DNA) failed due to this reason. I have discarded plates and cultures inoculated with contaminated pre-culture.
- **Making glycerol stocks of Kombucha-isolated Acetobacter strain -** Used 500ul of Kombucha-isolated Acetobacter culture, added 500ul of 50% glycerol. Stored in -80C.

HC

- **Checked on BC samples.**
    - -80C. Structure: stiff, melts incredibly quickly. Feel: hard.
    - -20C. structure: stiff, melts very quickly. Feel: like frozen leaves, malleable, but small elastic deformation before permanent shape change.
    - Leather hide: given to Victoria to do her fashion thing.
    - Air dry after dH2O. Structure: really brittle, shrunk a lot. Feel: stiff and looks red/brownish.
- **Dye treatment of BC.** Decided to try dyeing the cellulose with cotton blue (methyl blue), yellow food colour and green food colour. A small sample was immersed in 1000 ul of dye in a falcon tube.
- **Flow rate testing.** Tested the flow rate of the very thin cellulose filter. A 1.5ml eppendorf was set up with a filter bending slightly downwards. It filtered 250 ul in 4:48, flow rate of 52.1ul/min. Upon the second try, it filtered 210 ul in 4:21, with a flow rate of 48 ul/min
- **Treated first pellice of second batch.** The pellicle had grown into a poor structure containing many segmented circles of thicker cellulose connected with weaker thinner material. It was deemed useless for normal cellulose treatment and so it was decided to

blender it. Approximately two thirds of the pellice was blended, 197g total, along with 250ml of dH2O. The other third was stored in a labelled beaker containing dH2O for later analysis.

- **Treatment of blended pellicle.** All the cellulose was blended into a thick paste, this was then spread into shapes with a spreader and a loop
    - 2 coffee filters were covered with cellulose paste at different angles, oven dried at 70C
    - 2 coffee filters were covered with cellulose paste at different angles, oven dried at room temperature.
    - 1 pyramid and heart shaped by victoria, air dried
    - 1 iGEM logo by Ben, 70C oven dried
    - 1 Pingu shape, air dried
    - 1 jigsaw piece, air dried
    - 1 piece flat in oven 70C
    - 1 full plate of cellulose, oven dried 70C
    - 1 flat piece, freeze dried
    - 1 wet piece with green & yellow food dye, plus methyl blue.
    -

18/08/2014

HC

- **Checked on BC samples.**
    - -80C. Structure was,
- **Harvested pellicles from all trays with Gabi and Michael:** Harvested the pellicles from all the trays of the second batch. Sorted them into a large container of good pellicles and a smaller round container of bad pellicles with largely granular structure that worsened the mechanical properties of the material significantly.
- **Blended entire pellicle.** Made multiple samples, some figures, 4 water filters that are cone shaped, and some coloured samples.

MF

- **Testing ATCC23769 for contamination –** Plates grown for 48h at 30C on HS-agar displayed thick growth of colonies wit h E.coli – discarded the plates. Furthermore, liquid HS-media inoculated with supposed ATCC23769 displayed no cellulose production and were cloudy, indicating *E.coli* presence. Most likely, the strain sent from U.Röslig is not *Acetobacter*, but *E.coli*
- **Testing natural antibiotic resistance of Kombucha-isolated and U.Röslig's strain. –** Small HS media inoculated with Kombucha-isolated strain displayed small pellicles in cultures containing 1) no antibiotics, 2) kanamycin, 3) chloramphenicol, but no pellicle in ampicillin-containing media. HS media inoculated with U.Röslig's strain displayed no pellicles in any culture, however cultures with 1) no antibiotics and 2) kanamycin were

cloudy. This again points to *E.coli* (namely KanR one), however I will allow one more day of growth before making the final conclusion.

- **Harvesting bacterial cellulose from bulk production -** Harvested cellulose sheets from all cultures with HC. Most cultures contained a large amount of cellulose, and most were uncontaminated. However, with the exception of 9 cultures, cellulose was lumpy and mechanically weak. 9 cultures however, were thick and strong, without lumps. The reason for the difference is unknown. For cultures with aeration, no visibly higher celulose production was observed, however Acetobacter had grown around the aeration tubes. This could be due to poor aeration, the late stage at which aeration was applied, or due to ineffectiveness of aeration per se.

CBD/Functionalisation

- phosphatase of backbone in freezer, PCR cleanup
- digestion of PCR products with X+S

19/08/2014

HC

- **Observed structure of cellulose under microscope**
- **BC removal technique:** found that cellulose is much easier to separate from filter paper by moisturising the filter paper with distilled water and then peeling it off.
- **E. coli & acetobacter isolated from Kombucha co-culture.** Set up co-culture experiment with DH10B E. coli and Kombucha-isolated. Protocol followed was based on Master's thesis done on co-culturing:
    - Inoculate E. coli overnight in LB media, 100ul DH10B in 5ml of LB media to grow E. coli pre-culture
    - Inoculate Kombucha-isolated with Kombucha media for 48 hours in 30C incubater shaking at 50 rpm to form Acetobacter pre-culture.
    - Autoclave HS media and concentrated feedstocks solutions of
        - glycerol, glucose, sucrose, acetic acid and Kombucha Media
    - Make up HS media with carbon sources autoclaved to have a sterile media
    - Setup a 50 ml mastermix for each feedstock in a conical flask,
    - Add 150 ul of E. coli from the LB preculture to the conical flask
    - Aliquout 13 ml of the HS + E. coli mix into 15 ml Falcon tubes, repeat twice to obtain 3 filled Falcon tubes
    - Sterilise tweezers and scissors with bunsen. Then cut out a chunk of cellulose pellicle from the Kombucha preculture and add it to each of the three 15ml Falcon tubes.
    - Screw lids on loosely to allow aeration
    - Take OD samples of all samples,
    - Leave at static 30C incubation,

MF

- **Testing natural antibiotic resistance of Kombucha-isolated and U.Röslig's strain. –** Discarded all ATCC23769 containing cultures and plates, as cultures are contaminated. This is most likely due to E.coli contaminating the initial agarose stab received from U.Röslig.
- **Testing feedstocks for large-scale growth** - Set up Kombucha-isolated G.xylinus on different feedstocks (HS-glucose, HS-sucrose, HS-glycerol, HS-acetic acid and Kombucha tea medium) in 250ml conical flasks, using 50ml of each medium. All media set up in triplicates, standing, at 30C and left to incubate over several days.

CBD/FUNCTIONALISATION

- Phosphatase of backbone 9 and 16 that were digested with SpeI and XbaI: Heat inactivate restriction enzymes, use all DNA in phosphatase reaction (alkaline phosphatase), incubate 10min 37 degC, heat inactivate 2 min 75degC.
- Digestion of sfGFP, NiBP (inserts), CBDcex and CBDclos (backbones)
- Ligations:
    - SmtA, fMT, sfGFP, NiBP, dCBD into pSB1C3 backbone
    - sfGFP and NiBP with CBDcex and clos
    - Reactions used Invitrogen enzymes and aimed for ~30 fmol vector, ~90 fmol backbone.

20/08/2014

HC

- **Co-culture experiment OD readings.** Took OD readings of all co-culture samples and added additional Acetobacter chunks since no growth was seen with the initial liquid acetobacter medial added to Falcon tubes.
    - Plated 100 ul of selected samples on LB Kanamycin plates and HS plates with no antibiotic
- **Cellulose sample inspection**
    - The blended jigsaw piece was found to be moisture unstable, whereas the NaOH blended cellulose piece
    -

MF

- **Testing natural antibiotic resistance of Kombucha-isolated strain –** Strain grown since 14th August at 30C, 60rpm shaking. All cultures except one replicate of HS-Ampicillin contain visible amounts of cellulose, indicating that this strain is naturally resistant to all tested antibiotics (ampicillin, kanamycin, chloramphenicol) at the concentrations tested. Therefore, using this strain the requires either antibiotic concentrations. The strain could have increased resistance to antibiotics due to cellulose

production, which is known to naturally protect the cells from toxins, or due to antibiotic resistance genes in the chromosome or on some plasmids.

- **Testing growth of Kombucha-isolated G.xylinum strain on different feedstocks** – Visible cellulose present in all feedstocks (HS-glucose, HS-sucrose, HS-acetic acid, Kombucha tea medium) except HS-glycerol. By eye, largest growth is present in HS-glucose, HS-acetic acid and Kombucha tea medium. This implies that this strain can use acetic acid as the carbon source, as well as sucrose (to a certain extent). This can not be due to gluconeogenesis with energy derived from amino acids, as no cellulose was detected in HS-glycerol medium.

CBD/Functionalisation work

- Picked colonies for miniprep cultures for those transformations which worked from yesterday. Re-set up ligations and transformed for those that didn't work, including retransforming yesterdays ligations

General

- Prepared CCMB80 buffer as per openwetware recipe

21/08/2014

MF

- **Transforming Biobricks into E.coli DH10B -** 18 Biobricks in pSB1C3 (CamR) and 2 Biobricks in pSB1A2(AmpR) – requires 18 plates. 20 15ml tubes and 20 1.5ml microcentrifuge tubes. Prepared all of the necessary tubes and plates, labelled accordingly. Transformed the Biobricks in the evening, after returning from Thames Water purification plant. Used a chemical transformation protocol (see PROTOCOL) for transformation. Transformed 20 BioBricks in total, using plates with respective antibiotics and E.coli DH10B cells. Incubated at 37C standing.

CBD/Funcionalisaion

- Minipreps x 13 of sfGFP and NiBP in pSB1C3, CBDcex and CBDclos and nanodropped
- Set up colonies from yesterdays transformations - x28.

22/08/2014

MF

- **Transforming Biobricks into E.coli DH10B –** Growth was seen in all plates except for the one containing BBa_C0012 transformed E.coli. Inoculated 2 colonies from each plate into 15ml Falcon tubes containing 5ml LB-antibiotic (40-2=38 in total). Incubated at 37°C shaking (45° angle) overnight. NB! Growth room temperature may vary – recently temperatures between 35 and 39 have been reported.

- **Designing primers for pBla-Vhb-122 sequencing –**There is no full sequence available in any checked databases (GenBank, company websites). Designed primers based on *bla* promoter sequence to determine the layout of the plasmid so far.

- **Laboratory maintenance –** Prepared LB medium, cleared up lab, washed glassware.

CBD/Functionalisation

- minipreps from yesterdays cultures x 25 (3 were negative controls)
- Also sent registry parts PP1 and pBAD Arac-sfGFP for sequencing

23/08/2014

MF

- **Preparing chemically competent *E.coli* DH10B cells with XSM–** Used the protocol described in http://openwetware.org/wiki/TOP10_chemically_competent_cells
- **Miniprepping 20 Biobricks inoculated on the 22nd August -** Used Qiagen Miniprep kit following manufacturer's instructions with the exception of eluting with 30ul of EB buffer. Measured DNA concentrations using NanoDrop - concentrations were around 3ng/ul for almost all samples, meaning that minipreps were unsuccessful. A possible reason for this is bad growth of cells overnight, as shaking speed in incubator was low, and some cells had sedimented into the bottom. Re-inocculated 20ml of LB with single colonies from all samples, grew for 48+ hours at 37C shaking.
- **Measuring cellulose production of Kombucha-isolated Acetobacter grown on different feedstocks -** followed a protocol received from G.Buldum for measuring cellulose production - poured cellulose-containing cultures through a sieve to remove the liquid medium, placed all solid matter into a glass petri dish and left to dry at 80C oven for 48 hours.
- **Ordering primers for pBla-Vhb-122 sequencing –** Ordered primers iGEM39-42 from IDT.

XSM

- Made up 500ml CCBM80 media for chemically competent cells (see link below for recipe used)
- **With MF** made batches of chemically competent cells for DH10B and LEMO21 (BL21DE3).
- Details: followed protocol from http://openwetware.org/wiki/TOP10_chemically_competent_cells. DH10B had been cultured overnight in a 15ml falcon set up by MF. BL21 (DE3)

CBD/Functionalisation

- Yesterdays minipreps x 25 were nanodropped.

25/08/2014

XSM

- Digested 31 (K823012) with Spe1-HF and Pst1-HF for the interlab study. Incubated 45min 37degC. Heat killed 80degC 20min. Though apparently Pst1-HF can't be heat killed… probably should have done a PCR purification instead… Then phosphatased with rAPid. N.B noticed on 26/08/2014 that only added enough rAPid reaction components for 20ul of the digestion but added it to all 50ul of the digestion. Was incubated for 40min at 37degC (30min extra) and Heat inactivated 75degC 4min (2min extra). Allowed to reach RT before ligation.

- Ligation reactions: 31+17, 31+14, using previous digestions for 17 and 14 from 12/08/2014. Reaction set up in interlab doc. Note the 31+14 reaction would contain the wrong amount of 31 due to calculating total moles using volume of 25 instead of 55ul.

MF

- **Transforming *E.coli* DH10B cells with plasmid pBla-Vhb-122 received from Dr Lee -** Used chemical transformation protocol, incubated plates inverted at 37C.
- **Miniprepping 20 Biobricks inoculated on the 22nd August -** Growth room temperature 38.5C at the time of removal. For each culture, 20ml of LB were used in 48 hour incubations. Miniprepped the biobricks using Qiagen DNEasy kit, following manufacturer's instructions. Measured DNA concentration using NanoDrop - all cultures yielded good DNA concentrations, commonly around 200ng/ul.
- **Designing primers for Biobricks and pBAV1K-T5-gfp modification -** Designed primers for converting plasmids pSEVA351, pSEVA331, pSEVA311, pSEVA341 and pSEVA321 into Biobrick format. Ordered the primers.
- **Measuring cellulose production of Kombucha-isolated Acetobacter grown on different feedstocks -** samples left to dry at 80C for 48 hours were charred and stuck to the bottom; also residue nutrients from media had reacted to produce a sticky layer. For these reasons, it is not possible to weigh them, however as per visual inspection, the cellulose production from highest to lowest was the following:
    - 1)HS-glucose
    - 2)HS-glycerol
    - 3)Kombucha tea medium
    - 4)HS-acetic acid
    - 5)HS-sucrose (cellulose only present in one replicate)

LG

- **Assembling Vhb part into BioBrick Standard**

  1) PCR

  Amplifcation of pMA-T-101-34-Vhb with the set of primers designed on 11/08/2014:
  - Forward: 5' TCTAGAGTTTACAGCTAGCTCAGTCCT 3' Melting Temperature = 67.3
  - Reverse: 5' ACTAGTATTATTCTGCATCCTGTGCATACA 3' Melting Temperature = 67.2

  Forward and Reverse primers contain an XbaI and SpeI at their 5' ends, respectively. These "flappy" sites will allow insertion into BioBrick standard vectors.

PCR conditions for 1 sample
16.25ul H2O
5ul buffer
1ul forward primer
1ul reverse primer
0.5ul DNTPs
0.25 Phusion
1ul DNA

Setting up a negative control → Master Mix + no DNA

Master Mix for 3 samples (2 samples, called 1 and 2 + 1 control, called -c)
48.75ul H2O
15ul buffer
3ul forward primer
3ul reverse primer
1.5ul DNTPs
0.75ul Phusion
Add 1ul DNA

PCR cycle conditions
98 degrees for 30 secs
98 degrees for 10 secs
62-52 degrees for 20 secs (touchdown)
72 degrees for 40 secs
x35
72 degrees for 10 min
4 degrees

Gene Designer predicted a melting temperature of 67 degrees for the primers. However, lab label indicated a melting temperature of 58 degrees. Because the annealing temperature is advised to be set about 5 degrees lower than melting temperature, i set up a touchdown PCR for testing a range of temperatures between 62-52 degrees

2) Gel Electrophoresis
Verifying the final fragment size and also successful DNA amplification:

<u>Gel preparation</u>
50ml 1x TAE buffer
0.25g Agarose
4ul Sybr Safe

<u>Sample preparation</u>

10ul sample + 1.5 10x loading buffer
7ul 1kb ladder

→ Running the gel for 45 min at 110V

Visualization
504 (J23101+B0034+Vhb) + 14 (primer flappy ends) = 518 bp

→ Visible bands near the 500 bp band
→ Control shows trace of a band? will repeat PCR just in case

After two rounds of PCR (1 and 2, then samples 3 and 4:
1) Pooled samples into a single Eppendorf to produce samples 1-2 and 3-4
2) Added 0.5ul DpnI enzyme and incubated for 30min at 37 degrees
3) Carried out a PCR clean up and diluted in 25ul of dH2O
- Final DNA concentrations:
1-2 → 30 ng/ul
3-4 → 32.5 ng/ul

4) Digestion
Conditions:
12 ul DNA
2 ul Cutsmart buffer
1 ul XbaI
1 ul SpeI
4ul H2O

→ Final volume of 20ul
→ Incubating at 37 degrees for 2h
- Normally 1h should be enough. However, because the ends of the amplification primers did not contain a flap after the restriction site, this reduces the efficiency of digestion of XbaI, which normally requires about 2 bp on each side of the restriction site. To achieve successful digestion, therefore, we have to incubate for longer

5) Carried out a second PCR clean up and diluted in 25ul of dH2O
- Final DNA concentrations:
1-2 → 8.4 ng/ul
3-4 → 6.7 ng/ul

6) Ligation Calculations
→ Linearized vector (psB1C3), digested and dephosphorylated by Gabi, Xenia and Chris
→ Its final concentration is 35.6ng/ul (891ng per 25ul)
→ It is 2070 bp long

→ This vector is 4 times larger and also 4 times more concentrated than our insert. This balances itself out

→ To achieve a 2:1 molar ratio, you need double the amount of insert than vector. With the remaining 8.5 (10-1-0.5 referring to enzyme and buffer), I added 2.8ul of vector and 5.6ul of insert.

→ The negative control present 5.6ul of water instead of insert

→ Incubating overnight at room temperature

CBD/Functionalisation

- retransformation of dCBD

26/08/2014

LG

1. <u>Continuing cloning of hemoglobin gene into BioBrick Standard</u>

<u>7) Transformation</u>

<u>→ Three samples, called "260814 Vhb 1-2 transform", "260814 Vhb 3-4 transform" and "26082014 Vhb -c transform"</u>

→ 3ul ligation mix + 50ul competent cells (DH10B)

→ Incubate on ice for 15min

→ Heat-Shock: 45 seconds at 42 degrees

→ Samples back on ice for 2min

→ Add 500ul LB

→ Incubate at 37 degrees for 1h

2. Generation of the AraC-pBAD part

→ We would like to isolate the AraC and pBAD elements from part BBa_I746908

→ Designed primers as follows:

Forward primer anneals to suffix region and amplifies the vector backbone

Reverse primer anneals to 3' end of the pBAD promoter and amplifies the latter and the AraC coding sequence.

MF

Transforming BioBricks into *E.coli* – Transformed the following plasmids into *E.coli* using chemical transformation and E.coli DH10B cells:

1) plasmids containing BioBricks Bba_C0012 and BBa_I13600

2) SEVA plasmids: pSEVA pSEVA311, pSEVA321, pSEVA331, pSEVA341, pSEVA351

Incubated plates inverted at 37C overnight.

***E.coli* and *G.xylinus* co-culture experiment** - Prepared and autoclaved HS-glucose, HS-glycerol and HS-glucose-glycerol media. All samples were set up in triplicates.

XSM

- Transformed interlab study ligations. 200ul LB added in outgrowth. plated out all 200ul.

CBD/Functionalisation

- transformed dCBD in pSB1C3 ligation
- test digests of cloned fusions

27/08/2014

MF

Miniprepping BioBricks transformed on the 26th August – all transformed cultures had good growth, however growth room temperature was around 38-39C. Miniprepped plasmid DNA using Qiagen Miniprep kit, following manufacturer's instructions with the exception of dissolving DNA in EB buffer for 10 minutes before centrifugation. Measured DNA concentrations using NanoDrop. Stored samples in -20C freezer.

CBD/Functionalisation

- more Test digests of sfGFP/NiBP/SmtA/fMT constructs in pSB1C3 (BB16)/CBDcex/CBDclos and sending positives for sequencing
- additional miniprep cultures setup for constructs whose test digests failed

XSM

- Redigestion of biobrick 31 for interlab study g=to get new vector, gel purify. ligated with insert using both NEB and T4 ligase kits. See spreadsheet for exact reactions.

28/08/2014

XSM

- checked second batch IL study plates. Also discovered first batch of 31+14 transformants did actually have positive colonies after being left in incubator for longer fluorescence was visible more under the lightbox.

CBD/Functionalisation

- further test digests for unclear results
- digestion of arrived CBD constructs to put in pSB1C3
- minipreps of yesterdays cultures

29/08/2014

MF

Sequencing pBla-Vhb-122 - Submitted 2x5ul of 49ng/ul pBla-Vhb-122 DNA for sequencing using 5ul of 10uM forward primer iGEM39 and reverse primer iGEM40 each.

CBD/Functionalisation

- Ligation of digested CBDs into pSB1C3 backbone

30/08/2014

- Glycerol stocks made from 28/08 miniprepped cultures and plated out for plate stocks
- Transformation of new CBD arrivals into NEB 5 alpha

02/09/2014

MF

Culturing G.xylinum ATCC 53582 strain - Ben R. inoculated G.xylinum ATCC 53582 strain arrived from USA on the 1st September - the cultures (15ml HS in 50ml Falcon tubes) contained large cellulose pellicles,. Seeded two seed cultures (20ml HS in 50ml Falcon) with either liquid culture or the cellulose pellicle, grew standing at 30C.

Testing natural antibiotic resistance of ATCC 53582 and Kombucha-isolated (KI) G.xylinum using liquid media- Set up in total 62 cultures (20ml HS cultures in 50ml falcon tubes) containing either 1x Ampicillin, 4x Amp, 16x Amp, 1x Kanamycin, 4x Kan, 16x Kan, 1x Chloramphenicol, 4xCam, 16x Cam in triplicates, inoculated with either ATCC 53582 strain or Kombucha-isolated strain. Grew at 30C, standing. Also added three positive (no antibiotics in HS) and one negative (not seeded with bacteria) controls for both ATCC 53582 and Kombucha-isolated (KI) strains.

XSM

- set up miniprep cultures for IL study picking from plates from 26/08

CBD/Functionalisation

- Transformation plates from 28/08 and 30/09 had colonies picked for miniprep cultures, x4 each (CBDs inpSB1C3)

03/09/2014

CBD/Functionalisation

- Lab work is now documented in separate documents: Gabi&Chris shared document, and separate spreadsheets from XSM.

MF

Testing natural antibiotic resistance of ATCC 53582 and Kombucha-isolated (KI) G.xylinum - Set up in total 62 HS-agar plates (20ml HS-agar per plate) containing either 1x Ampicillin, 4x Amp, 16x Amp, 1x Kanamycin, 4x Kan, 16x Kan, 1x Chloramphenicol, 4xCam, 16x Cam in triplicates, inoculated with either ATCC 53582 strain or Kombucha-isolated strain. Grew at 30C, standing.

Testing cellulose productivity of ATCC 53582 and Kombucha-isolated (KI) G.xylinum strains - Prepared in total 38 cultures (50ml HS in 500ml conical flasks) containing HS medium. Seeded 18 cultures with ATCC 53582 and 18 cultures with KI strain by pipetting 20ul of seed culture into each flask. Added one neg. control for ATCC 53582 and one for KI strain (HS cultures not seeded with bacteria). Three cultures will be harvested from both ATCC and KI strains every day (triplicates), in total between days 1-6. Cellulose pellicles will be washed, dried and weight measured to quantify cellulose productivity of the strains respectively. Inoculated KI G.xylinum from culture 0815MFVa.

Plating out ATCC 53582 and KI-xylinum for single colonies - Prepared 4x HS-agar plates for ATCC and KI strains. Plated out 50ul of ATCC 53582 and KI strains, grew inverted at 30C. Samples 0903MFI - 0903MFIV.

Different feedstocks experiment for ATCC 53582 and Kombucha-isolated strain - Set up following media (20ml in 50ml Falcon tubes, 30C standing): HS-glucose, HS-sucrose, HS-acetate, HS-glycerol, Kombucha tea medium. Inoculated 3 cultures from each with Kombucha-isolated strain, and 3 with ATCC 53582 strain.

XSM
Miniprepped IL constructs.

04/09/2014

MF

-Cultures seeded on 02/09 OD600 still around 0, requires at least 24H more to grow it up.
-Temp around 30C in both growth chambers.

Testing natural antibiotic resistance of ATCC 53582 and Kombucha-isolated (KI) G.xylinum using plates- Have added three positive (no antibiotics in HS) and one negative (not seeded with bacteria) controls for both ATCC 53582 and Kombucha-isolated (KI) strains. Inoculated KI *G.xylinum* from culture 0815MFVa.
Measured OD600 of ATCC and KI strain seed cultures using Nanodrop - OD600 (ATCC)=0.056, OD600 (KI)=0.035. Plates will be plated with the same CFU from both ATCC and KI cultures to enable downstream comparison, CFU assumed to be proportional to OD600. Therefore, 50ul ATCC seed culture and 80ul of KI seed culture (0.056*50ul/0.035=80ul) were plated out respectively. No culture was added to negative control.

Testing cellulose productivity of ATCC 53582 and Kombucha-isolated (KI) G.xylinum strains - no visible cellulose was present in any of the cultures, thus sampling delayed one day for all samples

Extracting gDNA from Kombucha-isoalted G.xylinum strain - Inocculated 4x HS media in 50ml Falcon tubes with 5ul of culture from sample 0721MFI (samples named 0904MFIa-0904MFId). Used sterile technique and extreme care throughout:ethanol cleaned all surfaces and materials, used Bunsen burner and strippetes to pipette HS medium into 50ml Falcon tubes. Used a new, unopened bottle of HS medium.

05/09/2014

XSM

Set up SDM for fMT and SmtA to remove illegal rfc25 sites that were already in these parts. See spreadsheet for detailed reactions

MF

Testing cellulose productivity of ATCC 53582 and Kombucha-isolated (KI) G.xylinum strains - visible cellulose pellicles present, washed cellulose twice with distilled water (cultures removed at 15.00). Prepared 0.1M NaOH solution, washed cellulose pellicles in 0.1M NaOH solution for 1h at 60C.

Testing natural antibiotic resistance of ATCC 53582 and Kombucha-isolated (KI) G.xylinum using plates-
KI-strain: very small colonies present on both positive controls. None present in negative control. Very small, locally growing areas present in 1x Amp, none present in 4x Amp, 16x Amp. None present in any Cam plates. Some small, locally growing colonies present in 1 of 3 1xKan and 1 of 3 4xKan.
ATCC: Very small colonies present on 1 of 2 positive controls. None in negative control. No colonies present in any of Amp plates. No colonies present in any of Cam plates. No colonies present in any of Kan plates.

Different feedstocks experiment for ATCC 53582 and Kombucha-isolated strain - No visible growth present in any cultures, including positive and negative controls.`

Plating out ATCC 53582 and KI-xylinum for single colonies - very small, densely packed colonies present.

Testing natural antibiotic resistance of ATCC 53582 and Kombucha-isolated (KI) *G.xylinum* using liquid media- No visible growth present in any ATCC 53582 cultures, including positive and negative controls. KI-strain:small, visible pellicles present in 2/3 positive controls, none in negative control. Also very small pellicles present in 1x Cam, none in 4x Cam or 16xCam. No visible growth present in any Kan or Amp cultures.

PCR of pSEVA plasmids with primers iGEM57-60 for insertion of the Biobrick suffix and prefix- Used Phusion polymerase, set up master mix according to NEB recommendations; thermocycler protocol:
94C,3 min
94C, 30sec
69C, 30sec; -1C per cycle
72C, 5 min
X10
94C, 30sec
59C, 30sec
72C, 5 min

X25
4C hold

06/09/2014

XSM - re set up SDM PCR. see spreadhseet. Transform PCR products after DpnI digest directly.

MF

Double digest of 4 Anderson promoters+RFP coding devices (Bba_J23100, J23105, J23118, J23119) using EcoRI,PstI -
Reaction set up:
Plasmid DNA - 1ug,
EcoRI HF, PstI HF - 10U (0.5ul)
10xCutSmart - 5ul
DNA(J23100 - 260ng/ul) - 10ul
DNA(23105 - 107ng/ul) -4ul
DNA(J23118 - 135ng/ul) - 7.4ul
DNA(J23119-98ng/ul) - 10ul
Total reaction volume - 50ul
Digests incubated at 37C for 1H

DpnI digestion of PCR products from 06/09/14

- Added 1ul of DpnI from NEB to each of PCR tubes, incubated at 37C for 1h. According to some protocols in OpenWetWare, DpnI is active in PCR buffers - testing this out.
- Inactivated by incubating at 80C for 20 minutes

Gel electrophoresis of PCR products

- 1% agarose gel, added 3ul of SYBR Red dye to 50ml of agarose. Used 5ul of PCR product, added 1ul of 10x loading dye. Gels run at 90V for approximately 45 minutes, then visualized using a transilluminator with UV exposure time 3.5s. Ladders visible on the gel, but no PCR products - thus PCR reaction most likely failed, due to unknown reasons. However, there have been inaccuracies with pipettes, which could be a potential reason, or a contributor; the other is low number of cycles (25 after touch down) or high annealing temperature (59C) for these primers.

Gel purification of double digested biobricks J23100, J23105, J23118, J23119

- 1% agarose gel, 50ml of agarose used for pouring the gel, added 3ul of SYBR Red dye. Added 25ul of digested DNA (approximately 500ng), in 3ul 10x gel loading dyeGel run at 90V for 50 minutes. Then visualized using a blue-light box, and 1kbp fragments cut out using a razor blade.

Testing cellulose productivity of ATCC 53582 and Kombucha-isolated (KI) G.xylinum strains

- emoved 3 KI and 3 ATCC 53582 containing flasks from 30C, washed twice with distilled water, incubated in 50ml 0.1M NaOH for 1 hour at 65C. Then washed again twice with distilled water, poured off as much liquid as possible (through as sieve in order to avoid any loss of cellulose) and placed the flasks into 4C fridge until vacuum dessicator can be applied for drying and measuring the dry weight.

Testing natural antibiotic resistance of ATCC 53582 and Kombucha-isolated (KI) G.xylinum using liquid media

- **ATCC 53582 strain: No** visible growth observed in any cultures, including 3 positive controls. This could be due to longer time required for growth to become visible, or due to failure to inoculate cultures properly (bacterial cellulose tends to block pipette tips, which may have resulted in no culture carryover during inoculation)
- **Kombucha isolated strain: Pos. control:** growth present in 2 out of 3. Neg. control: no growth::
- No growth in any cultures, except very small, barely visible pellicles in 1 of 3 1xAmp and 1 of 3 1xKan HS media.

Different feedstocks experiment for ATCC 53582 and Kombucha-isolated strain

- **ATCC 53582 strain**: HS-glucose: visible, large clumps present in 3, HS-glycerol: medium-sized clumps in 3, HS-acetate: no visible growth, HS-sucrose: clumps present in all 3, Kombucha medium - small, coloured, fibrous cellulose present.
- **KI strain**: HS-glucose - small circular pellicles in 3, HS-glycerol very thin circular pellicles in 3, HS-acetate - no growth in any, HS-sucrose - very thin pellicles in all 3, Kombucha tea medium - thin,coloured fibrous pellicles present in all 3.
- No growth in any negative controls, with the exception of Kombucha tea medium, where fibrous coloured growth present - this most likely contamination of from the tea.

Testing natural antibiotic resistance of ATCC 53582 and Kombucha-isolated (KI) *G.xylinum* using plates

- **ATCC 53582:**
    - Positive control: dense growth. Neg control: no growth. Amp plates: no growth, Kan plates: no growth, Cam plates: no growth. No growth in any antibiotic plates with the exception of a few white, fluffy-looking colonies, which are likely contamination.
- **Kombucha-isolated strain:**
    - Pos. control :dense growth. Neg control: no growth.
    - Amp plates: relatively dense growth in 3 1x Amp (colonies very small, often fused, making colony counting very difficult). Peripheral growth in 3 4x Amp colonies. No growth in 16x Amp plates.
    - Chloramphenicol (Cam) plates: Growth in 3 1xCam plates. No growth in 4x Cam, 16x Cam plates
    - Kan plates: Peripheral growth in the edges in 3 1xKan and 3 4x Kan plates. No growth in 16x Kan plates. Peripheral growth could be due to partially lower

concentrations of antibiotic at the periphery of the plate, as antibiotic was pipetted into the centre of each plate and then stirred; the stirring might not have resulted in homogenous distribution of antibiotic for each plate

HC

Genomic DNA extraction of BL21 DNA:

- 5 ml of LB media was inoculated with 100 ul of BL21 overnight
- Heat the heat block to 56C
- Determine number of cells, an OD of 1 is equivalent to 1 x 10^8 cells/ml. Dilute media to an OD of 1, then measure out volume of 5 x 10^6 cells equivalent to a volume of 50 ul. Takes 3 volumes worth from each falcon tube.
    - Dilution process: measure OD of sample by pipetting 250 ul cells and 250 ul LB media to a cuvette. Based on OD, dilute to make up a solution of 250 ul containing both cells and autoclaved LB.
        - For sample 1: OD=5,628 so added 37.7 ul cells and 212.3 ul LB
        - Sample 2: OD=5,748 so added 37 ul cells and 213 ul LB
        - Sample 3: OD=5,916 so added 36 ul cells and 214 ul LB
    - Aliquoted 50 ul into 3 individual 1.5ml eppendorf temperatures for each sample
- Spin columns for 5 min at 4000 rpm
- Prepared a 200ml solution of PBS, based on 200ml dH_20 and 1 pellet from the prep room
- Resuspended pellet in 200 ul PBS
- Added 20 ul proteinase K to all 3 triplicates of each sample, pulse vortex mixed for 15s
- Incubated samples at 56C for 10 min in water bath
-

08/09/2014

HC

PCR of EFG and BAC plasmids with primers iGEM45-46 and iGEM49-50 respectively for extraction of genes from BL21 genomic DNA - Used Phusion polymerase, set up master mix according to NEB recommendations with two PCR triplicates using HF and one PCR triplicates using GC; thermocycler protocol: HC touchdown, One triplicates contains two samples with template and one without template as negative control.

Gel electrophoresis of PCR products

- 1% agarose gel, added 3ul of SYBR Red dye to 50ml of agarose. Used 5ul of PCR product, added 1ul of 6x loading dye. Gels run at 100V for approximately 60 minutes. Bands clearly

visible, ladder not visible for some reason. Speculated that too long loading time allowed diffusion of ladder, which explained lack of ladder. Bands of EFG was clearly smaller than EFG by about 350bp. All bands clear apart for EFG sample 6. All negative controls empty. Concluded that correct DNA had been amplified.

DpnI digestion of PCR products from 06/09/14

- Firealarm went off, but still added 1ul of DpnI from NEB for each of PCR tubes, pooled all 6 samples for BAC and 5 samples for BAC (discarded sample 6). Incubated at 37C for 90 minutes due to size of genomic plasmid. Pipetted up and down to ensure mixture of DpnI after 60 min.
- Inactivated by incubating at 80C for 6 minutes

QIAgen PCR cleanup

- Followed protocol, eluted with with 50 ul dH2O. Got good yields of 80 ng/ul and 89 ng/ul for BAC and EFG respectively. Stored in 'Miniprepped II + antibiotics' box.

XSM

- Colonies picked from transformations into miniprep cultures

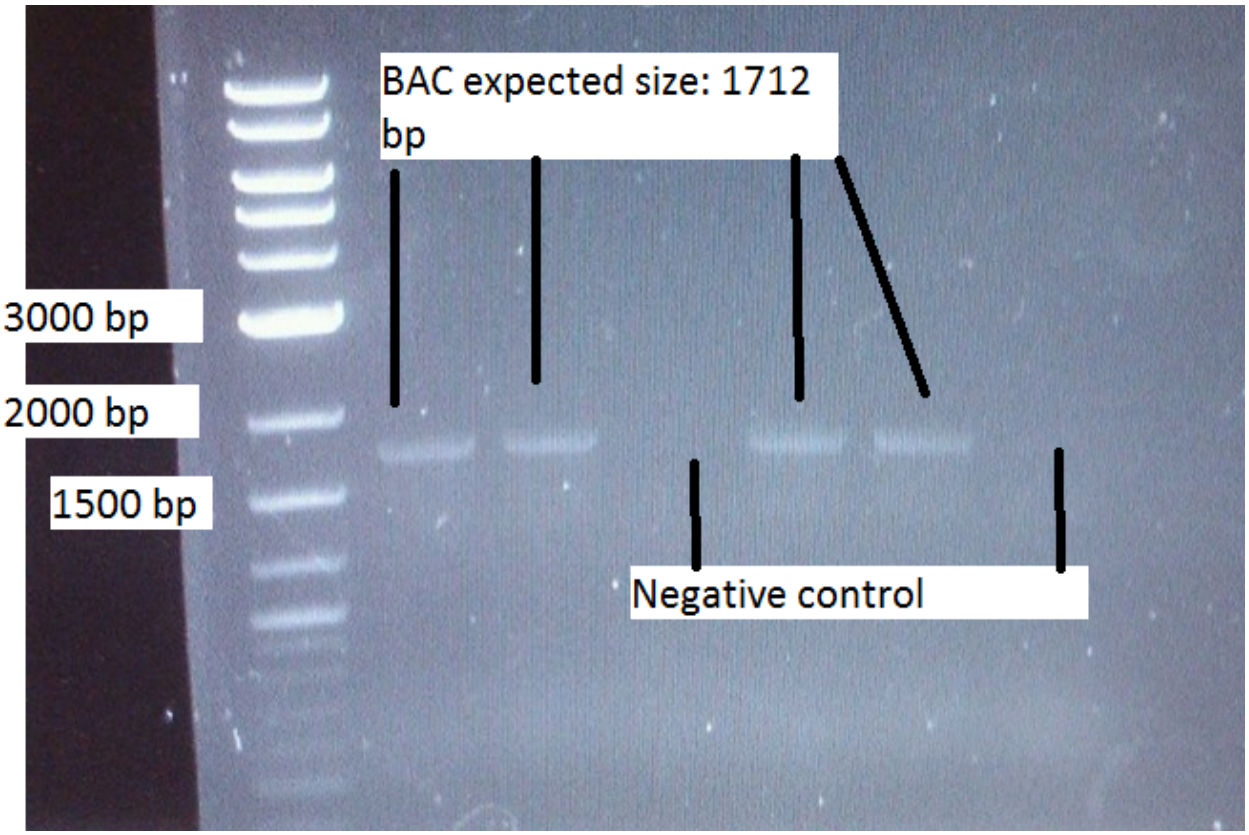
09/09/2014

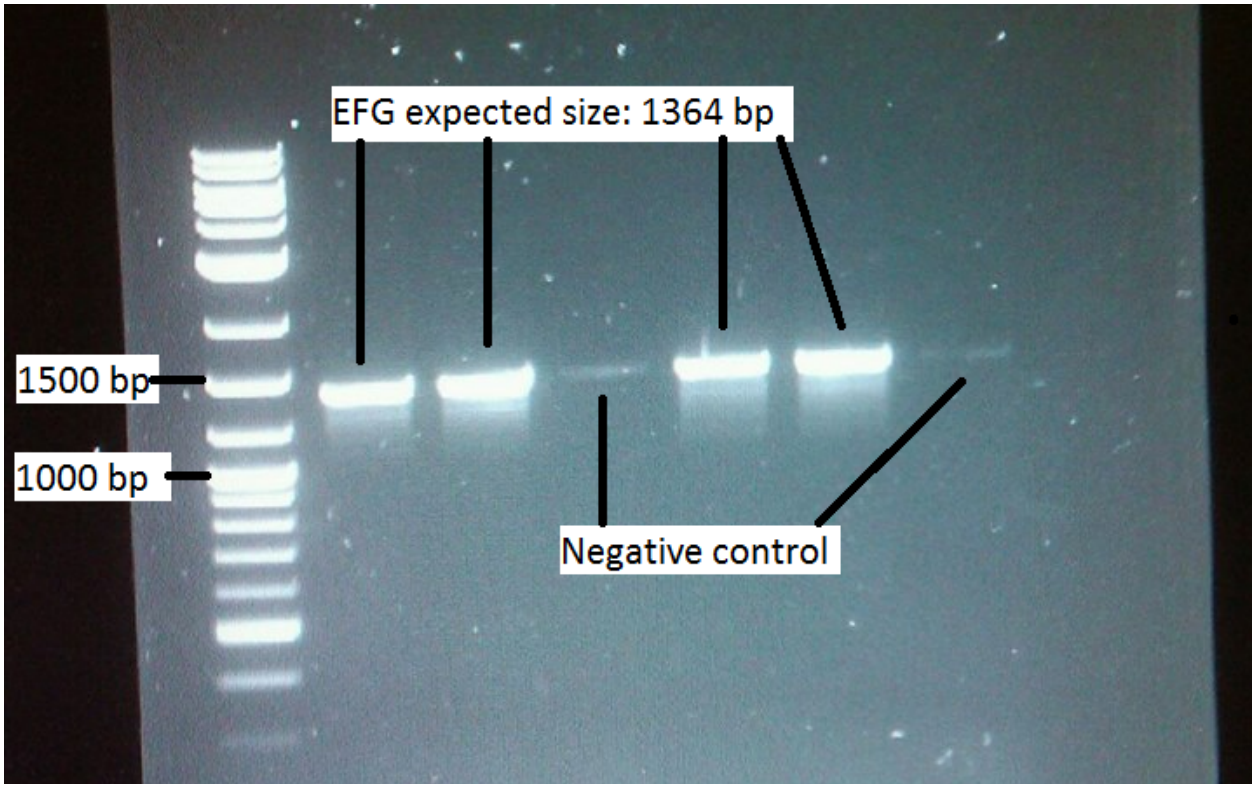
HC

PCR of EFG and BAC plasmids with primers iGEM47-48 and iGEM43-44 respectively for insertion of Gibson assembly sequences - Used Phusion polymerase, set up master mix according to NEB recommendations with one PCR triplicate using HF and one PCR triplicate using GC; thermocycler protocol: HC touchdown, One triplicates contains two samples with template and one without template as negative control.

Gel electrophoresis of PCR products

- 1% agarose gel, added 3ul of SYBR Red dye to 50ml of agarose. Used 5ul of PCR product, added 1ul of 6x loading dye. Gels run at 100V for approximately 60 minutes. Results shown below:


BAC expected size: 1712 bp
3000 bp
2000 bp
1500 bp
Negative control



- All bands were of expected size and usable.
- Pooled samples, all BAC together and EFG together. Added 4 ul DpnI to each eppendorf, 1 ul DpnI for each 50 ul samples.Incubated at 37C with start at 17:20. Finished DpnI treatment at 19:00, PCR purified with QIAgen protocol. Eluted in 50 ul water twice, did nanodrop, got good yields (around 80 ng/ul) and clear peaks at 260nm.

09/09/2014

HC

Large scale setup of cellulose production

Comparative production of cellulose in HS media

- Big vision: making cellulose sheets that can be used for the poster we are gonna present at iGEM.
- Triplicates of large tray growth: one that gets the aerosol treatment and one that does not. All static production.
    - 6 of Kombucha co-culture
    - 6 of ATCC 53582 alone
    - 6 of Kombucha Isolated strain alone
- Total of 18 trays

- Setup trays with

Setup of new HS media

- Durans were unavailable due to Collins using them. Set up 4 times 4500 ml of HS media.
    - Measured out the following ingredients for 5000 ml conical flasks:
        - 45g yeast extract
        - 45g peptone
        - 24.3g di-sodium hydrogen phosphate
        - 13.5 g citric acid
    - In a 2000ml conical flask, I measured out the carbon source, which was in this case glucose:
        - 180 g glucose
    - Added 3500 ml distilled water to the 5000 ml conical flask and 1000 ml distilled water for the 2000 ml conical flask.

Green tea media using Suzanne Lee's protocol with sucrose (common sugar)

Materials:
- 1x200ml organic cider vinegar
- 200g granulated sugar
- One piece of live Kombucha culture
- 2x tea bag

Procedure:
1. Boil 2L of water to 100degrees
2. Pour into container
3. Add green tea and allow to brew for 15minutes
4. Remove tea bags
5. Add sugar (200g)
6. Stir until dissolved
7. Cool down to below 30degrees
8. Add organic cider vinegar (200ml)
9. Add one piece of live Kombucha culture. It will sink to the bottom of the container
10. Cover growth container with breathable cloth and grow at 25 degrees (oven)
11. Fermentation starts after 48-72h, thin skin and bubbles will be produced and culture will come back out on the surface
12. When product becomes about 2cm thick, take it out
13. Wash with soaped water
14. Let dry

10/09/2014

XSM -

- Reset up more SDM reactions.
- Transformations for interlab study constructs

11/09/2014
XSM - set up some IL study growth cultures

12/09/2014
XSM - IL study test run with help of CA

13/09/2014
XSM - MoreSDM set up - Gabi and Chris

15/09/2014
XSM - actual IL study culture set up for full measurement
16/09/2014
XSM - IL study measurements with help of CA

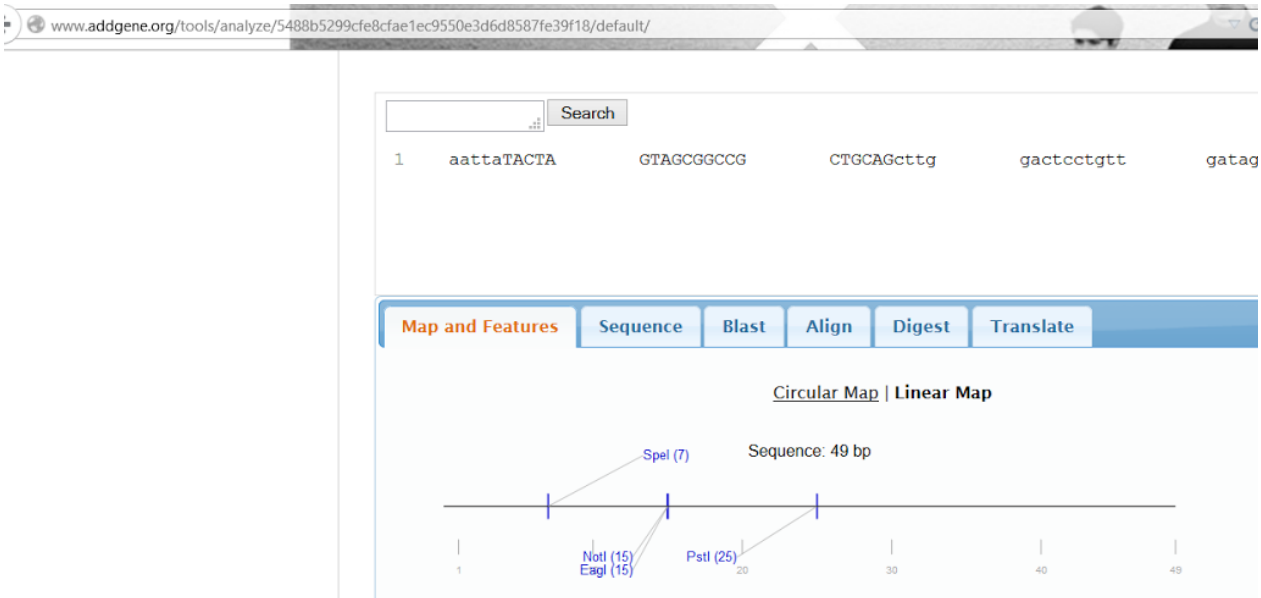
-

18/09/2014

HC

DpnI treatment and PCR purify of pSEVA plasmids 311,321,331,341,351.

SpeI digestion of vectors PCR amplified by primers 75-76



- PCR amplified vectors used: 311 I & III, 321 I, 331 I & III, 341 I & III, 351 I & III,
- Set up single ligations

sample name	311 I	311 III	321 I	331 I	331 III	341 I	341 III	351 I	351 III

DNA conc, ng/ul	166	102	182	73	195	104	66	100	58
DNA vol, ul	29.0	29.0	29.0	29.0	29.0	29.0	29.0	29.0	29.0
SpeI, ul	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Cutsmart, ul	8.0	4.9	8.8	3.5	9.4	5.0	3.2	4.8	2.8
Water, ul	80.2	49.3	88.0	35.3	94.3	50.3	31.9	48.3	28.0
total volume	118.3	84.2	126.8	68.8	133.7	85.3	65.1	83.2	60.8
Mastermix of water and cutsmart added	88.3	54.2	96.8	38.8	103.7	55.3	35.1	53.2	30.8

Mastermix		10% extra
water	505.6	556.1233
cutsmart	50.6	55.61233

- Left for digestion for 2.5 hours.
- Heat inactivated at 80C for 20 min in the heat block
- **Tomorrow:** PCR purify with MinElute columns and elute in 15 ul water only

Phosphorylation

- First step: 20 ul total volume containing
    - 1 ul PNK, 2 ul Ligase buffer, 17 ul DNA,
- Labelled with DNA as
    - 1 = 311 II, 2 = 311 IV, 3 = 321 II, 4 = 321 IV, 5 = 341 IV, 6 = 351 II, 7 = 351 IV,
- Heat inactivated at 80C for 20 min in the PCR machine
- 19/09/2014: set up PCR tubes containing:
    - 4.1 … 4.7: 4 ul DNA mix from step 1, 1 ul Ligase buffer, 1 ul ligase, 14 ul dH2O
    - 8.1 … 8.7: 8 ul DNA mix from step 1, 1 ul ligase buffer, 1 ul ligase, 10 ul dH2O
    - 2.1 .... 2.7: 2 ul DNA mix from step 1, 1 ul ligase buffer, 1 ul ligase, 16 ul dH2O

20/09/14

DK

Setup culture trays with Suzanne Lee's protocol:

Materials:

- organic cider vinegar
- granulated sugar
- One piece of live Kombucha culture
- Green tea bag
-distilled water

Procedure:

1. Pour distilled water into 5000ml or 2000ml conical flasks, up to 4500ml or 1800ml respectively
2. Cover the tops with foil and tape
3. Boil 2L of water to 100 degrees, in the autoclave.
4. Spray with antibacterial spray, and set up trays for growth
5. Pour into container
6. Add green tea and allow to brew for 15 minutes, 1 tea bag per liter of autoclaved water
7. Remove tea bags
8. Add sugar, 100g per liter.
9. Stir until dissolved
10. Cool down to below 30degrees
11. Add organic cider vinegar 100ml per liter of green tea solution,
12. Open bag of living Kombucha culture, take pellicle, hold with blue roll
13. Add one piece of Kombucha culture pellicle by cutting a chunk with scissors. It will sink to the bottom of the container. Be careful not to add any liquid Kombucha media.
14. Cover growth container with blue roll, be careful not to lean over the trays and keep sterile conditions.
15. Fermentation starts after 48-72h, thin skin and bubbles will be produced and culture will come back out on the surface
16. When product becomes about 2cm thick, take it out
17. Wash with soaped water
18. Let dry

Notes:

1. 36 liters distilled water put in the autoclave at 16:55, 10 liters HS ready from previously; 4 litres of water boiled using the kettle.

2. Tray information:
   Tray no. 1-8: 2.25L/tray Kombucha media
   Tray no. 9-14: 1.5L/tray Kombucha media
   Tray no. 15-16: 4L/tray Kombucha media
   Tray no. 17-19: 4L/tray HS media
3. Two pieces of ATCC53582 pellicle cake was cut into 10 pieces and 9 pieces respectively and are distributed into the 19 trays.
4. ATCC53582 pellicles (around 3cm^2 each) are added to the media once the temperature has dropped below 30 degree.
5. (!) No pellicle observed after 72 hours of fermentation.
6. Thin layer of cellulose is observed in tray no. 19 (HS media) on 25/09; all others: none. Possibly the cells was concentrated on the portion of cake seeded to no. 19.
7. Re-seed (ATCC 53582) the tray no. 1-18 (no. 17 HS media is contaminated) on 25/09 using liquid-cultured cells (in HS media). 0.1ml culture liquid was distributed to 17 trays respectively.
8. Cell culturing: inoculate cell in autoclaved HS media. 150ml/flask. Culture the cells in 30 degree C incubator for 48 hours.

LG -

1. Heat inactivating enzymes from yesterday's digestion
   - SpeI = 80 degrees for 20min
   - PstI = 80 degrees for 20 min
   - XbaI = 65 degrees for 20min
2. Dephosphorylating vector backbones with Roche rAPid Phosphatase
   2.1. Split 10ul digestions into two 5ul samples, then added 2ul buffer, 1ul enzyme and 2ul H2O to each
   2.2. 30min incubation at 37 degrees
   2.3. Enzyme Inactivation at 80 degrees for 2 minutes
3. PCR-clean up of linearized backbones

XSM

Bits and pieces of lab work for various people. Running gels, gel extractions, minipreps

21/09/2014

LG -

1. Transformation of pSB-Vhb ligation mix into DH10B
   1.1. 3ul pSB-VhB mixed with 50ul competent DH10B
   2 samples:
      - pSB-Vhb
      - pSB1C3 (empty vector control
   1.2. Incubated on ice for 15 minutes
   1.3. Heat-shock for 45 seconds at 2 degrees
   1.4. Back on ice for 2min

1.5. Added 500ul LB
1.6. Incubated for 1h at 37 degrees (shaking conditions)
1.7. Centrifuged at 8000rpm for 5min
1.8. Discarded 300ul LB
1.9. Plated the remaining 250ul on a chloramphenicol resistant Agar plate
1.10. Incubate overnight at 37 degrees

2. Preparing mini-prep cultures

2.1 Selected 9 colonies (labelled 1-9) from the 3 different pSB-AraC-pBAD plates (all expected to contain the same construct, but this was built using three different templates)

2.2 Also prepared acsAB and acsCD overnight cultures from the glycerol stock

22/09/2014

LG -

1. Mini-prepping in the morning
   1.1. Sweet yields obtained
2. Digested all 9 pBAD samples with EcoRI and PstI
   2.1 Digestion conditions per sample (10ul total volume):
   - 5ul sample
   - 2ul Cutsmart
   - 0.5ul EcoRI
   - 0.5ul PstI
   - 2ul H2O

   2.2 1h Incubation at 37 degrees
   2.3 Running a gel
      2.3.1 100ml TAE, 0.5g Agarose, 8ul Sybr Safe
      2.3.2 Addition of 1ul 10x Loading Dye to each sample
      2.3.3 8ul of 2-log DNA ladder
      2.3.4 100V for 1h
      2.3.5 Sweet positive colonies!
   2.4 Sending pBAD 1 and pBAD 2 for sequencing
      2.4.1 5ul sample + 5ul H20 to dilute samples down to roughly 100ng/ul
      2.4.2 two tubes: BAD 1 and BAD 2, each containing 10ul (5ul for forward and 5ul for reverse reaction). Primers: 10ul VF and 10ul VR (prepared by Gabi)

23/9/2014

LG -

1. Mini-prepping Vhb 1-10 samples and AcsAB samples
2. Nanodrop - good yields
3. Restriction digest of Vhb samples

3.1. 5ul sample, 1ul cutsmart, 0.25 of each enzyme (did colonies 1-5 with PvuII and EcoRI, and 6-10 with NcoI and SpeI),

XSM - test digested PCRd pSEVA samples for MF and HC