Cellulose Cross-Linker

Project Journal

- 5/28/14
  - Found BioBrick of CBM: http://parts.igem.org/Part:BBa_K863111
  - Found BioBrick of Strep: http://parts.igem.org/Part:BBa_K283010
- 5/29/14
  - Researched a possible project approach: synthesize CBM-GST and Strep-GST fusion proteins.
    - Theory: GST is a tag which allows for purification once the protein has been translated.
    - GST forms dimers; if we synthesized both in the same cell, we should get a mix of CBM-GST-GST-CBM, CBM-GST-GST-Strep, and Strep-GST-GST-Strep
      - will spatial characteristics of protein production influence the distribution of dimer products?
    - CBM/CBM homodimer would provide cross-linking strength
    - CBM/strep heterodimer would allow for modular addition of parts
    - strep/strep would not bind to the material's surface
    - purify set of GST-tagged proteins and rub onto surface
  - Another approach would involve synthesizing a set of "traditional" fusion proteins
- 6/2/14
  - Decided to synthesize DNA: CBD-strep-CBD with $(GGGGS)_3$ linkers
  - Contains inducible T7 promoter and RBS
- 6/10/14
  - Decided to also get E. coli cells to express an AviTag on their surface (using membrane localization signal OmpA found in 2012 distribution kit). This will allow them (once biotinylated) to attach to the cellulose surface coated in recombinant protein. Ordered overhanging primers to ligate parts together (which we will attach tomorrow) and as soon as they arrive will start ligating.

- 6/12/14
    - Streptavidin protein purification workflow:
        - Run through iminobiotin agarose gel, eluting non-strep lysate
        - Elute streptavidin fusion protein with acid at pH 4
        - Neutralize and reconstitute protein
- 6/13/14
    - Ran PCR to amplify out fusion protein DNA
        - Unsuccessful (womp)
- 6/16/14
    - Reran PCR
        - increased annealing temp from 62° to 65° to increase specificity
        - used .5x of 1:20 primer dilution
    - Performed gel extraction of 1.3kb pdt
        - Nanodrop: 4.4 ng/µL
- 6/17/14
    - Ran restriction digest of DNA + BioBrick plasmid (3H in 2014 distro)
        - used XbaI and SpeI
    - Ligated parts together and transformed into NEB 5-alpha competent E. coli
    - Incubated in SOC for 1h, plated to incubate overnight at 37°
- 6/18/14
    - Both plates have colonies!
    - Made replicate plate and performed colony PCR to see which colonies had the correctly-ligated plasmid
    - Colony PCR gel was pretty ugly. Run by Kosuke? or redo tomorrow, perhaps
- 6/19/14-6/20/14
    - Backbone religated itself so we mini prepped to get the plasmid out
    - Mini prep was successful with a concentration of 48.9 and a 260/280 of 2.01
- 6/23/14
    - Digested miniprepped backbone plasmid and performed digestion cleanup
        - Nanodrop: 23.7 ng/µL of linear backbone
    - Ligated fusion protein gene into backbone, transformed 5-alpha cells
        - Plated 20/200 µL on two plates, incubated overnight at 37°
- 6/24/14
    - Ran colony PCR on new plates
    - COLONY 5 IS OUR HERO
        - Inoculated liquid culture, incubating overnight
- 6/25/14

    - Miniprepped plasmid (nanodrop: 23 ng/µL)
        - Sent to Elim for sequencing
    - Transformed T7 Express lysY competent E. coli and made two plates
- 6/26/14
    - Transformation successful! Prepared 10 mL pre-culture and incubated at 30°C
- 6/27/14
    - Cultured in 200 mL LB + chlor at 10:30 am, waiting 5 hours to get cloudy
    - ELIM sequencing results were weird…..
        - PCRing colony 5 + miniprepped plasmid to see if insert is there
            - Band was most likely due to semi-coiled plasmid :(Alaina will repeat procedure from Tuesday's colony PCR
- 6/30/14
    - Reran colony PCR
        - Unsuccesful :(
            - 3 of 8 colonies showed nothing
            - 5 of 8 showed vector with no insert
- 7/1/14
    - Tried digestion and ligation again
    - Transformed into 5-alpha cells, incubated over night on three plates
        - 20 ul, 50 ul, 200 ul
- 7/2/14
    - Transformation efficiency was really really bad. 20 ul and 50 ul had no colonies and 200 ul had only 6 colonies total
    - This is most likely because the backbone had to be incredibly diluted in order for the ratio of insert to vector to be 3:1 since the concentration of insert is only 4.4 and we only had a small volume left.
    - Did colony PCR on the six samples and which once again showed only empty vectors :(
    - Realized this was because I forgot to digest the insert and only digested the vector (lol whoops).
- 7/3/14
    - Trying everything again
    - Need more insert DNA so PCRed this successfully with now two samples.
        - One has a concentration of 99.6 ng/ul
        - The other 96.7 ng/ul
    - Redid digestion and clean up and ran on a gel
        - Insert digested correctly, but backbone perhaps looks a little weird? Will reevaluate on Monday and if everything is good, will ligate and transform.

- 7/7/14
    - Used linearized backbone from distribution kit and PCRed insert for ligation
    - Ordered primers SB-prep-3P1 and SB-prep-2Ea to apmlify out more backbone from random plasmids.
    - Gel of digest looks good
        - Backbone 5.9 ng/ul
        - Insert 79.2 ng/ul
    - Proceeding to ligation
        - 3 ul backbone (18 ng DNA)
        - 0.5 ul insert (35.1 ng DNA)
    - Plated transformed 5 alpha cells
        - one plate 200 ul
        - one plate 20 ul
- 7/8/14
    - Really bad transformation efficiency-only 2 colonies total
    - Doing colony PCR and making replicate plate
    - Made more LB +chlor plates
    - gel indicated ligation failed :(
    - PCRing out more backbone from GFP via colony PCR with SB primers (1:20 dilution).
        - Goldtaq protocol used
        - Annealing temp 55 degrees
- 7/9/14
    - PCRing more backbone did not work :(
    - Trying PCR again this time with miniprepped plasmid from the esterase group
    - Increased annealing temp to 60 degrees C
    - Gel was successful indicating a 2 kb product
- 7/10/14
    - PCRed backbone concentration: 130.7 ng/ul
    - Digested backbone and insert and did clean up
        - Backbone concentration: 39.0 ng/ul
        - Insert concentration 63.1 ng/ul
    - Ligation complete
        - 1.28 ul backbone (50 ng)
        - 1.55 ul insert (97.5 ng)
    - Transformation of 5 alpha cells
        - 50 ul 1 plate
        - 150 ul 1 plate
- 7/10/14
    - Retransformed NEB 5-alpha cells with CBD insert
- 7/11/14

    - transformation looked good with numerous distinct colonies on both plates
    - ran colony PCR on 8 colonies (4 from each plate)
        - VF2 VR primers
        - Annealing temp 57 degrees
    - Colony #1 is correct lenth. Colonies 2-7 show no insert. Replicate plate was made.
- 7/17/14
    - Did colony PCR of LysY transformed cells
    - Colonies 2+3 have insert, will begin liquid culture on Monday
- 7/21/14
    - Going back to colony 1 from 5-alpha transformation – confirmed by colony PCR
        - Will miniprep plasmid tmrw and submit for sequencing!
- 7/22/14
    - Sequencing data was bad
    - Miniprepped colony 1
        - concentration 109.1 ng/ul
- 7/23/14
    - Sequencing data was heinous
- 7/24/14
    - Ordering primers for middle of CBD insert so colony PCR is more accurate
        - Expected lengths of products
            - VF2/VR: 1.2 to 1.3 Kbp
            - VF/Bridge R: 800 bp
            - Bridge F/VR: 800 to 900 bp
            - BridgeF/Bridge R: 450 bp
    - re-cultured colony number 1 to submit for sequencing
- 7/25/14
    - doing PCR of original CBD DNA from IDT
        - Using Q5 mastermix and corresponding protocol
        - Gel was bad. Incorrect bands everywhere.
    - miniprepped liquid culture of colony 1 to submit for sequencing
        - concentration 60.3 ng/ul
        - submitted for sequencing
    - Redoing PCR with temperature gradient and less primers
        - Q5
        - Samples 1 and 2
            - Annealing temp: 66 degrees
        - Samples 3 and 4
            - Annealing temp: 68 degrees
        - Samples 5 and 6

            - Annealing temp: 70 degrees
        - Samples 1,3 and 5 have 2.5 ul of forward G block forward primer and 2.5 ul of G block reverse primer
        - Samples 2,4 and 6 have 1.25 ul of the above primers
        - Gel looked really good with 2 and 4 being the cleanest bands. Will look further on monday.
- 7/28/14
    - Did PCR Cleanup of all samples
        - Concentrations
            - 1: 47.6 ng/ul
            - 2: 31.3 ng/ul
            - 3: 30.5ng/ul
            - 4: 90.9 ng/ul
            - 5: 45.3 ng/ul
            - 6: 64.2 ng/ul
    - Digested 6 samples, ligated into chlor backbone, and transformed 5-alpha cells.
        - Digestion reactions (using EcoR1 and PTS1)
            - 1. 21 ul DNA, 5 ul water, 3 ul cutsmart, 1 ul enzymes
            - 2. 29 ul DNA, 6 ul water, 4 ul cutsmart, 1 ul enzymes
            - 3. 29 ul DNA, 6 ul water, 4 ul cutsmart, 1 ul enzymes
            - 4. 11 ul DNA, 6 ul water, 2 ul cutsmart, 1 ul enzymes
            - 5. 22 ul DNA, 4 ul water, 3 ul cutsmart, 1 ul enzymes
            - 6. 15 ul DNA, 2 ul water, 2 ul cutsmart, 1 ul enzymes.
            - Backbone. 5 ul DNA, 3 ul water, 1ul cutsmart, 1 ul enzymes
        - Digestion Cleanup Concentrations
            - 1. 60.6 ng/ul
            - 2. 25.8 ng/ul
            - 3. 28.5 ng/ul
            - 4. 13.6 ng/ul
            - 5. 24.1 ng/ul
            - 6. 39.6 ng/ul
            - BB1. 32.3 ng/ul
            - BB2. 39.0 ng/ul
        - Ligation reactions
            - 1. 1.5 ul insert DNA, 1.5 ul BB1 DNA, 2 ul water, 5 ul sticky end mastermix
            - 2. 3.4 ul insert DNA, 1.5 ul BB1 DNA, 5 ul sticky end mastermix
            - 3. 3.1 ul insert DNA, 1.5 ul BB1 DNA, 5 ul sticky end mastermix

                - 4. 6.4 ul insert DNA, 1.3 ul BB2 DNA, 7.8 ul sticky end mastermix
                - 5. 3.7 ul insert DNA, 1.3 ul BB2 DNA, 5 ul sticky end mastermix
                - 6. 2.2 ul insert DNA, 1.3 ul BB2 DNA, 1.5 ul water, 5 ul sticky end mastermix
            - Fire alarm may have messed up transformation. Competent cells and ligation mastermix were left out for an hour

- 7/29/14
    - Minimal growth on chlor plates; allowed to incubate an extra day.
    - Realized that LysY cells (for T7 promoter expression) are chlor-resistant, so transformed samples 1, 2, 3, and 6 into amp backbone and plated.
        - 1. 0.9 ul insert DNA, 6.5 ul sticky end mastermix, 5.6 ul amp BB1
        - 2. 2.0 ul insert DNA, 7.6 ul sticky end mastermix, 5.6 ul amp BB1
        - 3. 1.9 ul insert DNA, 7.5 ul sticky end mastermix, 5.6 ul amp BB1
        - 6. 1.3 ul insert DNA, 6.9 sticky end mastermix, 30.1 ul amp BB2
    - Plated 150 ul on LB + Amp + IPTG plates

- 7/30/14
    - No growth on amp plates; allowed to incubate an extra day.
    - All chlor plates (except for #3) had growth! Ran colony PCR to screen for insert using VF2/VR and Bridge primer sets
        - Gel was bad. Rerunning VF2/VR set with 57° instead of 59° annealing temp, along with positive control of 600bp insert
        - 2nd gel still showed nothing. Since the cells took more than 1 day to grow, colonies were most likely contamination and transformation was not successful.
- 7/31/14
    - PCR IDT original construct
        - 2 samples to be gel extracted
        - 2 to be cleaned up
        - 1 to be submitted for sequencing
    - Q5 mastermix protocol
        - Annealing temp 67 degrees
        - 1.25 ul each primer (G block) used instead of 2.5
    - Decided to do clean up of all samples
        - Concentrations
            - 1. 84.0 ng/ul
            - 2. 58.4 ng/ul
            - 3. 64.6 ng/ul

                - 4. 54.1 ng/ul
                - 5. 94.3 ng/ul
            - submitted sample 3 for sequencing because its concentration and purity was the best
            - PCRing more backbone from cellulase plasmid (chlor) and promoter/RBS (amp)
                - Q5
                - Annealing temp 64 degree
                - Gel looked weirdddd (tons of wrong bands)
- 8/1/14
    - Sequencing results look okay but kinda weird
        - First cellulose binding domain looks good then it isnt a good match
        - probably because there is a primer site in the middle of our gene
        - proceeding with digestion/ligation/transformation
    - Gel extracting samples 1 and 2
        - final concentration: 64.0 ng/ul
    - Digestion (E and P restriction enzymes)
        - Gel extract: 15.6 ul DNA, 2 ul cutsmart, 1 ul enzymes, 1.4 ul water
        - 3. 15.5 ul DNA, 2 ul cutsmart, 1 ul enzymes, 1.5 ul water
        - 4. 18.5 ul DNA, 2.5 ul cutsmart, 1 ul enzymes, 3 ul water
        - 5. 10.6 ul DNA, 2 ul cutsmart, 1 ul enzymes, 6.4 ul water
        - Amp Backbone. 8.4 ul DNA, 2 ul cutsmart, 8.6 ul water
    - Digestion clean up concentrations
        - Gel extract. 1.8 ng/ul
        - 3. 19.3 ng/ul
        - 4. 21.4 ng/ul
        - 5. 6.5 ng/ul
        - BB amp. 9.1 ng/ul
    - Ligated 3 and 4. Gel extract and 5 had too low of concentrations
    - Ligations
        - 3. 4 ul BB, 3.2 ul DNA, 1 ul t4 buffer, 0.5 ul ligase, 1.3 ul water
        - 4. 4 ul BB, 4.8 ul DNA, 1 ul t4 buffer, 0.5 ul ligase
    - Transformed into 5 alpha cells
        - out of SOC so used LB instead and incubated for 3 hours
        - Cells spun down and all but 200 ul media removed
            - resuspended and all cells plated
- 8/4/14
    - Both plates grew! Ran colony PCR on 8 colonies
    - Nothing showed up on colony PCR :(
- 8/5/14

    - Miniprepped promoter RBS part to use for its backbone
    - combined all PCR cleanups from IDT CBD construct
    - ran digestions and ligation using t4 ligase and its protocol
    - transformed into 5-alpha cells
- 8/6/14
    - plates grew.
    - ran colony PCR with Bridge F/ Bridge R primers
    - Nothing showed up on gel
        - discovered we ordered the reverse primer in the forward sequence. OOPS
        - ordered corrected bridge r primer
- 8/8/14
    - PCRed more CBD from IDT with gBlock primers
    - Digested CBD and amp backbone
    - Did gel extraction after digestion
    - Ran ligation
- 8/12/14
    - Only one colony grew
    - Ran colony PCR and it looks like insert may be there
    - Make liquid culture to miniprep and submit for sequencing
- 8/13/14
    - Liquid culture didn't grow. Grew in the wrong antibiotic. Oops
    - Religated CBD with old gel extracted digestions
    - Transformed into 5 alpha
- 8/14/14
    - 16 colonies grew
    - Running colony PCR on all
    - 1,7, and 8 have potential inserts
    - Made liquid cultures
- 8/18/14
    - PCRed more IDT CBD construct with new primers
    - Digested and cleanup product
    - Made liquid cultures from CBD replicate plate 1, 7, and 8
- 8/19/14
    - Ligated CBD into amp backbone
    - Miniprepped colonies 1,7 and 8 and submitted for sequencing
    - Transformed 1,7, and 8 into T7 Cells
    - Out of SOC so used LB and glucose instead.
- 8/21/14
    - Sequencing for CBD looks bad
    - Doing colony PCR from plate with ligation from CBD amplified using new primers

    - Colony PCR failed...Kosuke says its because there were too many cells. Liquid culturing 1,5,7 and 8 to redo PCR tomorrow
- 8/22/14
    - Transformed OmPA from 2012 Distribution kit 22 p and plated on chlor plate
    - Redid colony PCR
        - 1,5 show bands at 3 kb???
        - 7 and 8 show nothing
        - Kosuke is designg primers for the middle of the sequence and will redo on monday. If this doesnt work, will get synthesized from DNA 2.0
- 9/12/14
    - Resynthesized CBD with two different cellulose binding domains to avoid problems of homologous recombination.
    - OmPA did not grow

9/13/14

Digested, ligated and transformed the new construct (after PCR) with E and P restriction enzymes into PSB1A3 backbone then into LysY e. coli

9/15/14

Colony PCR confirmed the insert was present in 5 colonies

9/16/14

Submitted for sequencing

9/18/14

-Sequencing was good

-Growing 3 ml precultures overnight at 37 C to start protein induction

9/19/14

- Added 3ml precultures to 100 mls LB +Amp

-Incubated for 4 hours at 37 C

-Added 1000X ITPG to induce protein

-Will incubate at room temp for 48 hours

9/24/14

-Completed HisTag Extraction

-Correct band did not appear :(

-Will retry inducing protein