Laboratory Records: Cloning of Chrysene Degradation Construct and Biohydrogen Synthesis Construct

Team CCA_San_Diego iGEM 2019

Purpose

- Cloning and experimentally validating chrysene degradation by the Chrysene construct (Part:BBa_K2566006) and hydrogen synthesis by the HSU-ETCD construct (Part: BBa_K3270002)

DNA Source

Synthesis of Fragments

- Insert fragments were synthesized by IDT and Twist Bioscience and were delivered to us lyophilized.

DNA Preparation

- Vials containing the lyophilized DNA (1000 ng) were spun down, and 100 µL UltraPure Distilled Water were added to the lyophilized powder.

- Tubes were closed, vortexed, and incubated at 60°C for 15 minutes to resuspend the DNA. Aliquots were made for cloning, and stocks were stored at -20°C.

Cloning of Chrysene and HSU-ETCD Constructs

Originally, we planned for the inserts to be digested and ligated using Golden Gate Cloning; however, due to low efficiency yields, we decided to try Gibson cloning.

PCR

Chrysene

The following Gibson assembly primers were designed for Chrysene:
Note: anneal regions are noted in green

Primer Name		GC %	Tm	Anneal pair at
pSB1C3-T forward	TTATATACTAGTAGCGGCCGCTGCAGTTACTTCGCGTTATGCAGGCTTCCTC	52	65	
psb1C3-T reverse	CTCTGCCTCGTGATACGCCTGTTGATCGGGCACGTAAGAGGTTCC	56	65	60
Ins1-F	AGGCGTATCACGAGGCAGAGAATTCGCGGCCGCTTCTAGATTTACAG	50	66	
Ins1-R	CTAGGCCTCCCACTCTAGTACATGCATGTCATCAACGCTCCCCA	54	65	60
Ins2-F	GAGCGTTGATGACATGCATGTACTAGAGTGGGAGGCCTAGCTTCC	56	64	
Ins2-R	AGTTCCCTCTAGTAGTACGTAGTCATCACGAAGCAGGTGAGAAGCA	50	65	60
Ins3-F	TCTCACCTGCTTCGTGATGACTACGTACTACTAGAGGGAACTCAATTGAAATTAAGGAGG	40	63	
Ins3-R	CCTGCATAACGCGAAGTAACTGCAGCGGCCGCTACTAGTATATAAAC	48	63	58

HSU-ETCD

Primer Name		GC %	Tm	Anneal pair at
HSU-1F	TAGGCGTATCACGAGGCAGAGAATTCGCGGCCGCTTCTAGATTTACAG	50	64	
HSU-1R	TAGATTACTCTAGTACATGCATGTTATTAGATAAGGGCTGATTTATCGATCCCAAGACGC	41	65	59
HSU-2F	TACTAGAGTAATCTACCATTAAGTTAAGGAGGTTTTTTAATGATTCCAGAGAAGCGTATC	33	64	
HSU-2R	ATATACTCTAGTAGTACGTAGTTATTAATCCTCCAAGTCACCACAGAAACGATATCCTTC	40	66	59
HSU-3F	ACTACTAGAGTATATAATATAATTAAGGAGGTTTTTTATGGCCATGAGTGACGAAGCAAT	41	66	
HSU-3R	TGCAGCGGCCGCTACTAGTATATAAACGCAGAAAGGCCCACCCGAA	44	65	60

Note: pSB1C3-T forward and psb1C3-T reverse primers from the Chrysene Gibson primers table were used for HSU-ETCD assembly.

Primers were ordered from IDT as Custom Oligos.

Primer Resuspension

1. Briefly centrifuge the tubes before opening them.
2. Resuspend the oligos in nuclease-free water to a concentration of 100 µM.

PCR Protocol

1. Dilute gBlocks 1:10 by adding 1 µL of gBlock to 9 µL ddH2O.
2. After resuspending primers according to the IDT, make primer working stocks by pipetting 2 µL of forward primer and 2 µL of reverse primer to 16 µL ddH2O.
3. Make master mix of 22:25 of ddH2O:PrimeSTAR Max DNA Polymerase, depending on how many reactions are run.

4. Add 47 µL of master mix to each PCR tube while on ice.
5. Add 1 µL appropriate gBlock to each PCR tube.
6. Add 2 µL of each appropriate primer mix to each PCR tube.
7. Flick the PCR tubes to mix and spin them down.
8. Place tubes into a thermal cycler:
    a. 98°C for 10 sec
    b. Annealing temp (59°C) for 20 sec
    c. 72°C for 30 sec extension (largest gBlock is 2.7 kB, polymerase does 1k every 10 seconds)
    d. 25 cycles total

Primer pairs for Chrysene PCR:

1. Ins1-F + Ins1-R
2. Ins2-F + Ins2-R
3. Ins3-F + Ins3-R
4. pSB1C3-T-F + pSB1C3-T-rev2

gBlock templates for Chrysene PCR:

1. Ins1
2. Ins2
3. Ins3
4. psB1C3

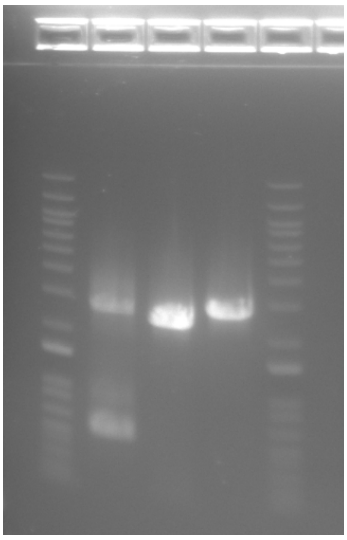
Primer pairs for HSU-ETCD PCR:

1. HSU-1F + HSU-1R
2. HSU-2F + HSU-2R
3. HSU-3F + HSU-3R
4. pSB1C3-T-F + pSB1C3-T-rev2

gBlock templates for HSU-ETCD PCR:

1. HSU-ETCD1
2. HSU-ETCD2
3. HSU-ETCD3
4. psB1C3

PCR reactions were then run on an E-gel and column purified using the Zymo Clean and Concentrator kit. A Nanodrop was used to find the concentration of DNA for each fragment.



HSU-ETCD Insert 1, 2, and 3 after PCR; because Insert 1 showed multiple bands, the band of expected size was extracted through gel extraction.

Gibson Assembly

1. Determine [DNA] for each fragment.
    a. Use the Biotools calculator to convert ng/µL to fmol based on fragment size and Nanodrop output.
2. Dilute the PCR fragments to 30 fmol in nuclease-free water in new tubes.
3. Add 1 µL of each fragment to a PCR tube and add an equal volume of 2x NEB Gibson Assembly Mix (4 µL, in this case).
4. Add 2 µL for a final reaction volume of 10 µL.
5. Mix by flicking tube and spin down.
6. Incubate at 50°C for at least one hour or 30°C overnight.

Transformation

Chemically Competent Transformations

1. Use NEB5a, and add 1 µL of undiluted assembly mix. Incubate on ice for 30 mins.
2. Heat shock at 42°C for 30 seconds.
3. Place on ice for 5 minutes.
4. Add 270 µL SOC media to cells and incubate at 37°C with shaking for 1 hour.
5. Plate 50-100 µL on selection plates and incubate overnight at 37°C.

Note: for BL21 cells, follow the Chemically Competent Transformations protocol, but heat shock for only 10 seconds.

Electrocompetent Transformations

1. Add 20 µL ddH2O (1:3 dilution) to reactions and mix.
2. Transform 1 µL of diluted reaction into EPI300 *E. coli* using the electroporator.

Cultures were plated on chloramphenicol plates at 25 µg/mL.

Plasmid Screening

Resulting colonies were then screened to ensure that they contained the correct plasmid, with all of the inserts.

Colony PCR

1. Make primer working stocks by pipetting 3 µL of forward primer and 3 µL of reverse primer to 24 µL ddH2O.
2. Each reaction should have 12.5 µL SapphireAmp Fast PCR Mix (2x Premix), 1 µL primer mix (1:10 dilution), and 11.5 µL ddh2O. Swatch a colony from the plates onto a pipette tip, place in PCR tube, and multichannel to mix.

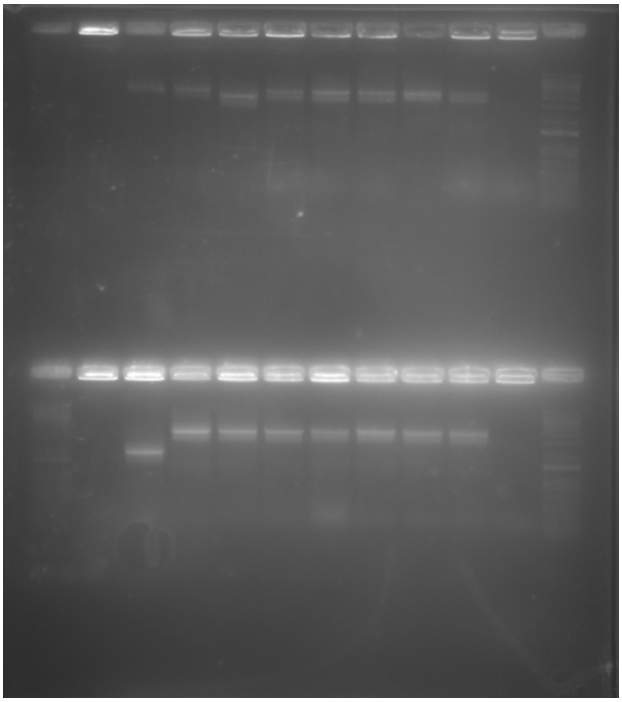
The following primers were designed for Colony PCR.
Chrysene:

		% GC	Tm
C_PSB1C3-R	GCTCACATGTTCTTTCCTGCGTTATCCC	50	64
C_PSB1C3-F	GGAACCTCTTACGTGCCCGATCAAC	52	65
C_Chry-F	CCCTGAAAGTGGATTGGCCTCATGAGA	52	65
C_Chry-R	CTTTAATGCGTTCCGCCAGTGCCA	54	66

HSU-ETCD:

		% GC	Tm
C_HSU-F	CCTTCGCCAGCAAATGATGCGTTTAATGAG	47	65
C_HSU-R	TTAGGCATCTTCCCGCTAAGGTCGT	52	65

Note: C_PSB1C3-R and C_PSB1C3-F were used for HSU-ETCD as well.

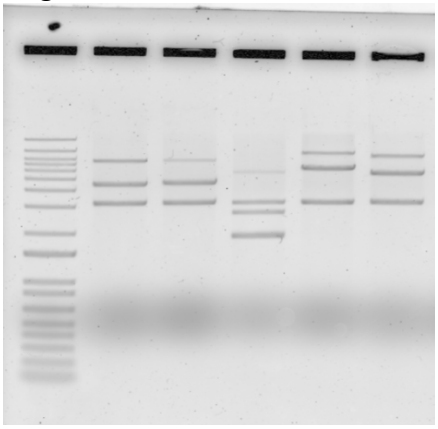


Colony PCR for Chrysene Construct.

Restriction Map

1. Select 2+ restriction enzymes that cut the target plasmid into fragments of varying sizes.
2. Perform a 25 µL restriction digest with the restriction enzymes and corresponding buffer.

Below is an image of the chrysene construct, which was cut with XhoI and PsiI to produce fragments of 3.1 kb and 6.4 kb. The largest band is undigested plasmid.



Plasmid Miniprep and BL21 Transformation

Following verification of plasmid through Colony PCR and Restriction Mapping, plasmids were miniprepped using the QIAprep Spin Miniprep Kit and transformed into BL21 cells.

Analysis of Clones

Chrysene

A 2.5 mg/mL stock solution of chrysene was prepared by dissolving chrysene into DMSO under a fume hood. The solution had to be slightly warmed on a 37°C heating block for the chrysene to fully dissolve.

A 96-well plate was set up with M9 minimal media and chrysene at concentrations of 0 µg/mL, 10 µg/mL, 20 µg/mL, 30 µg/mL, 40 µg/mL, and 50 µg/mL. The chrysene plasmid-transformed strain and a BL21 strain containing PSB1C3 were diluted to an OD600 of 0.01 and added for a total volume of 200 µL in each well. The plate was incubated in an incubated shaker at 37°C. A plate reader was then used to measure OD600.

HSU-ETCD

H2Blue drops, which are blue but turn clear in the presence of dissolved hydrogen, were analyzed using a scanning fluorometer to create a spectra. The optimal wavelength for absorbance was determined to be 650 nm. Similar to the chrysene assay, a 96-well plate was set up with M9 minimal media and H2Blue drops at varying concentrations (1:100 drop, 1:10 drop, 1 drop, 2 drops, and 3 drops; a “drop” was defined to be 13 µL of H2Blue). After incubating in an incubated shaker at 37°C, a plate reader was used to measure the OD650.