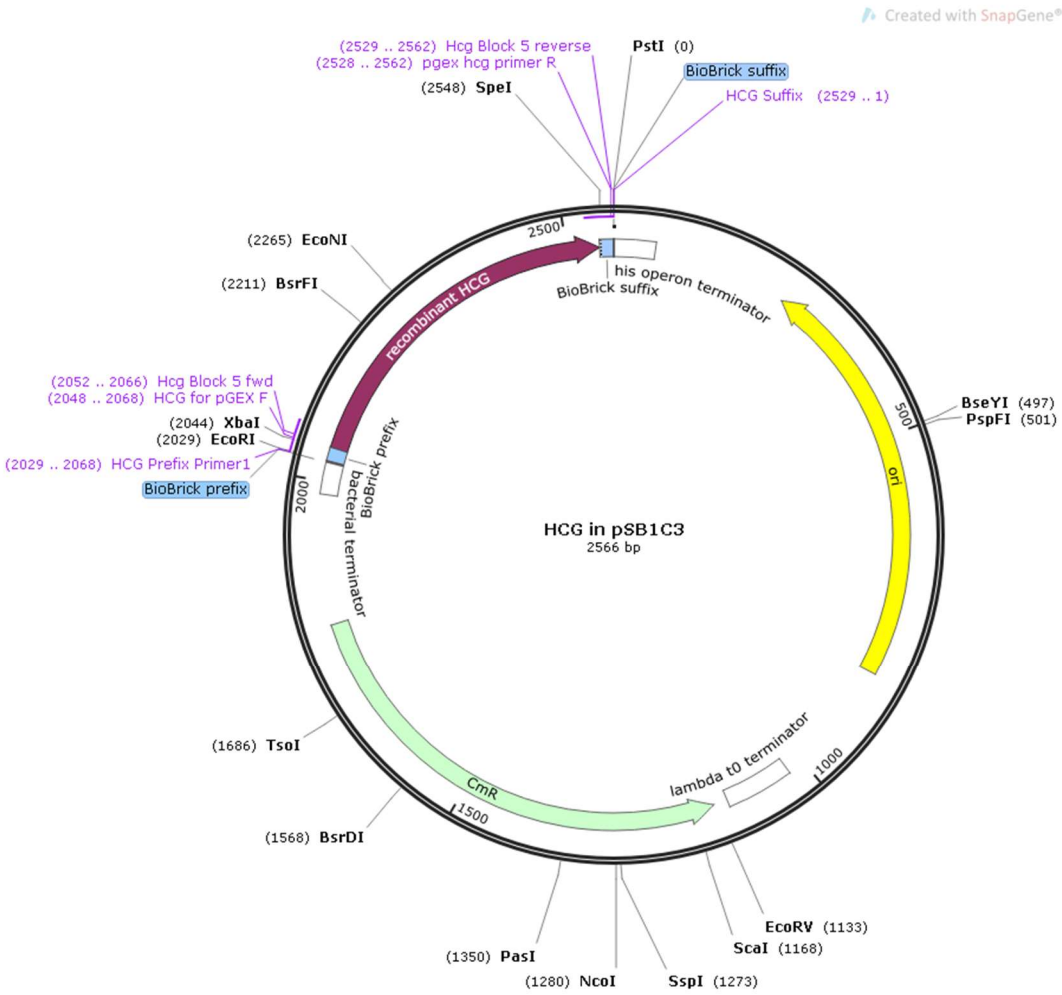
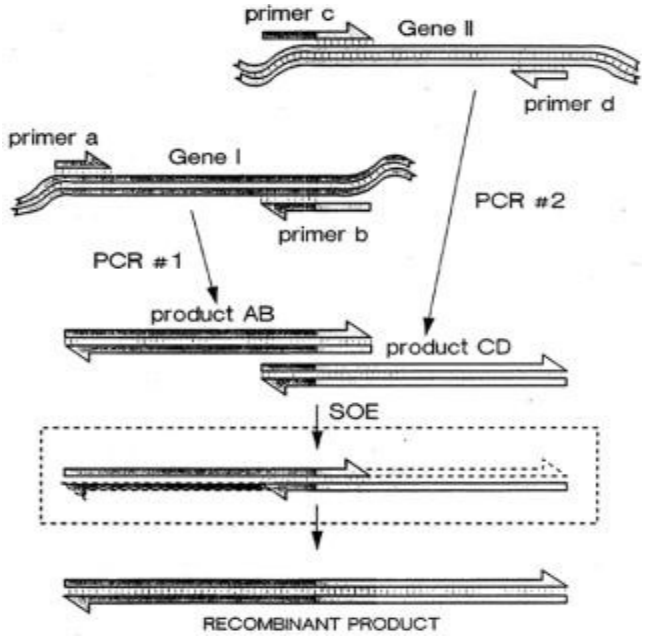


8/9-8/14
LIGATION hCG to pcb1c3
Transformation
OVERNIGHT
COLONY PCR
MINI PREP
DIGEST -- GEL

OVERLAP EXTENSION PCR

Due to the expense of purchasing the entire Factor C sequence as a new part and the opportunity to attempt a method that has never been done in the Georgia State University iGEM lab Factor C is planned to be synthesized via overlap extension PCR. A modified protocol written by Ichiro Matsumura was used. This method uses PCR to recombine DNA sequences instead of using restriction sites. By using a 3'-end primer that matches each template block and a 5'-end primer that matches a part of the block sequence and a part of the new block sequence. The two blocks can be recombined using DNA polymerase. The basic mechanism for overlap begins with a PCR to generate the two fragments (AB and CD) that have ends modified by mispriming so that they have homologous regions. Through mixture, denaturation, and reannealing the AB 3'-end will anneal onto the 3'-end of the bottom strand of the CD. Extension of the overlap product is used to form the recombinant product (the overlapping ends of the fragments are created by primers b and c while a and d match the individual fragments). The method can be repeated to add together more than two DNA fragments.
Below is a figure to illustrate the mechanism described above.



7/ 20 Primer Designs for Overlap Extension PCR

Primers were designed to fit the top strand on the 5’-end of one block (ex. Block 1) and the 3’-end bottom strand of Block 1 is taken and reverse complemented to fit the 3’-end of the bottom strand of the next block (ex. Block 2), this process is repeated until all primers are completed. A total of 8 primers were designed for 5 blocks. (Annealing temperature were taken into consideration thus all primers temperature were within range of one another).

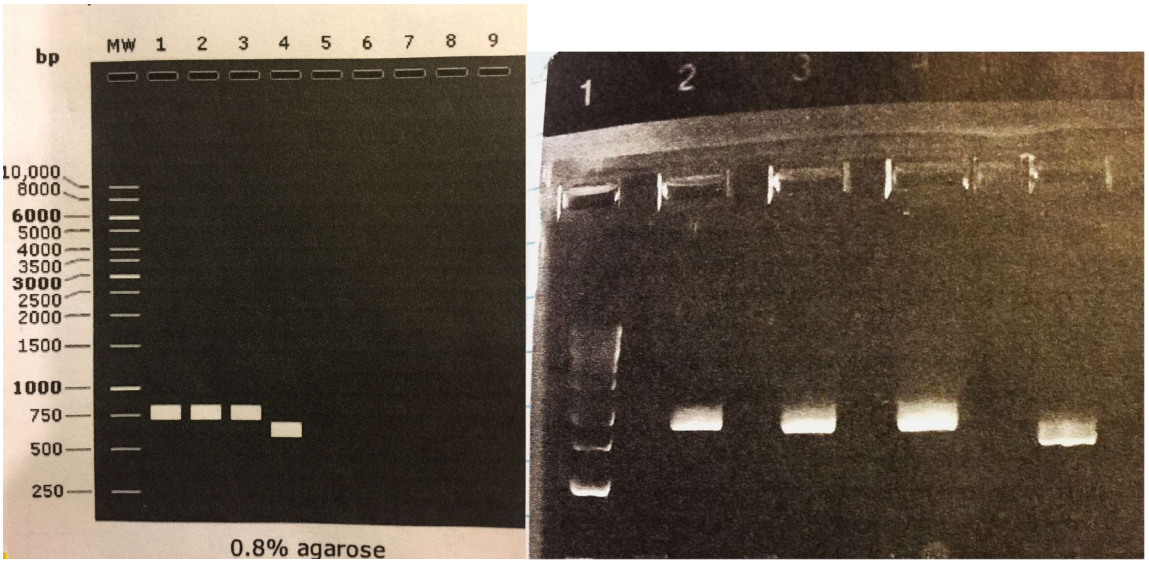
8/14 Preparation of G-blocks for Overlap extension PCR

Factor C was divided into 4 G-blocks and ordered from IDT. The overlap extension PCR requires that each block have the right ends annealed onto them. The blocks were diluted to a 10ng/ul then diluted further to 1ng/ul. After the forward and reverse primers were added to each block through PCR.

Protocol:
Each of the amounts from the following table was added to tubes 1 through 4. Normal thermocycler conditions were used.

Tube 1	Tube 2	Tube 3	Tube 4
25ul Master Mix	25ul Master Mix	25ul Master Mix	25ul Master Mix
2ul Block 1	2ul Block 2	2ul Block 3	2ul Block 4
2.5ul Forward Primer Block 1	2.5ul Forward Primer Block 2	2.5ul Forward Primer Block 3	2.5ul Forward Primer Block 4
2.5ul Reverse Primer Block 1	2.5ul Reverse Primer Block 2	2.5ul Reverse Primer Block 3	2.5ul Reverse Primer Block 4
18ul Nuclease-free water	18ul Nuclease-free water	18ul Nuclease-free water	18ul Nuclease-free water

Results:



A simulated gel was created and then the actual sampled of each were run on an e-gel. 5ul of product with 1ul of 6X dye and 14 ul of DI water was added to each well.
The wells include the following from left to right: Gene Ruler 1kb DNA ladder, PCR of g-block 1, PCR of g-block 2, PCR of g-block 3, PCR of g-block 4.

Discussion:

The bands are near the reference points on the molecular weight ladder that signifies the bands are the correct size. The fourth block has a lower number of base pairs than the three other blocks.

8/15- Preparation for Overlap extension PCR

Materials:

- Quigen 2x mastermix
- PCR product of G-block 1 DNA
- PCR product o fG-block 2 DNA
- PCR product of G-block 3 DNA
- PCR product of G-block 4 DNA
- Phusion Polymerase
- 5x reaction buffer for phusion DNA polymerase
- dNTP mix (10mM)
- DMSO
- DnP1 restriction enzyme (targets methylated sequences and cleans DNA)

Protocol:

Part 1: 100ul total reaction was made and split into the reaction and a negative control. In a PCR tube the following was mixed: 30ul PCR block 1, 30ul PCR block 2, 20ul of reaction buffer, 2ul of dNTP, 14ul of nuclease free water. The reaction was divided into two tubes, 49.5 in each. .5 ul of the phusion DNA polymerase was added to only the reaction tube. Thermocycler conditions used are depicted in the table below. After PCR 5ul of each reaction was taken out order to run a diagnostic gel and .35 ul (10 units) of Dnp1 was added to all 4 tubes and incubated at 37□ for 1 hour.

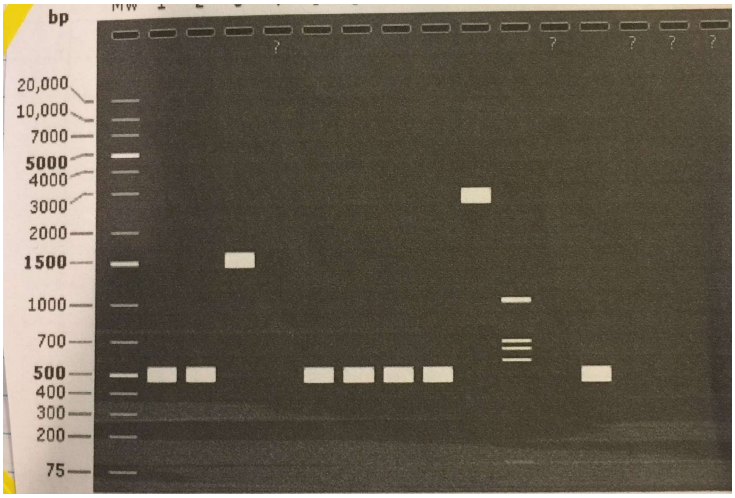
# Cycles	Step	Temperature in □	Time
1	Initial denaturation	100	1 min
18-22	Denaturation	95	50sec
	Annealing	60	50s
	Extension	68	3 min
1	Final Extension	68	3 min

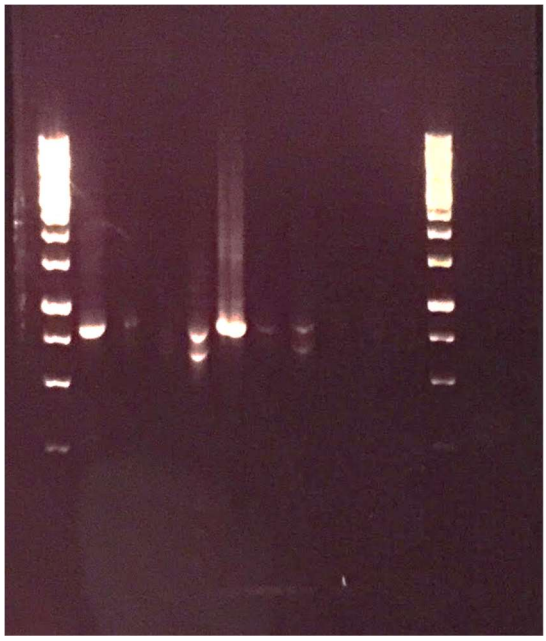
Part 2:

A second PCR reaction was preformed in order to amplify the products from part 1. 2ul of forward primer 1 and reverse primer 2 were added to the both tubes that contained blocks 1 and 2. 2ul of forward primer 3 and reverse primer 4 was added to both tubes that contained blocks 3 and 4. Thermocycler conditions are noted in the table below. 5 ul was taken out of each tube in order to run a diagnostic gel.

# Cycles	Step	Temperature in □	Time
1	Initial denaturation	98	1 min
12	Denaturation	95	30s
	Annealing	55	30s
	Extension	68	1 min
1	Final Extension	68	5 min

Part 3:
Each sample was purified using a qiagen pcr purification kit. Part 1 was repeated but the products from 1-2 and 3-4 were added into the same tube in place of the individual block dna. which was divided into a reaction and negative control. 5ul of the each was taken out to run on a diagnostic gel.
Each sample taken from the samples were run on a 1% agarose gel.
Results:
Simulated Gel:





Gel of each 5ul sample taken from each part of the protocol. Wells contain the following: Gene Ruler 1kb plus molecular weight ladder, Reaction of blocks 1 and 2, Negative control of blocks 1 and 2, Reaction of blocks 3 and 4, Negative control of blocks 3 and 4, Reaction of 1 and 2 with 3 and 4 products, negative control of reactions 1and 2 with 3 and 3 and 4 and lastly Gene Ruler 1kb plus molecular weight ladder.

Discussion:
The first reaction of blocks 1 and 2 did not proceed the band is near the 400kb line which is the original length of the G-blocks.The negative control did not show a bright band, there is a small dim band showing the presence of DNA but not amplified DNA. Blocks 3 and 4 did not combine nor amplify as there are no bands, however the negative control shows 2 bands one of each fragment of DNA. Since the original reaction of 1 and 2 did not take place when amplified the band shows the same length as the band in well 1 and a very slight increase of the negative control. The reaction of 3 and 4 amplified shows dim bands due to the fact the bands were amplified by the primers but not recombined. The overall combination of reactions of blocks 1 and 2 with 3 and 4 did not show up on the gel.

REASONS this did not work? NEED help explaining!
Desciption of primer design of HCG for PGEX