Date:20190323 Location: Haidian Experimental Middle School iGEM Laboratory

Persons involved: Harry Fang, Annie Xian

Goals: Construction of PSB3K3-LuxR-sfGFP plasmid

Experiment steps and results:

-PCR amplification of PSB3K3-LuxR-sfGFP plasmid

-Gel Electrophoresis and extraction of PCR products

-Failed PCR amplification (Wrong length for LuxR sequence)

Date:20190324 Location: Haidian Experimental Middle School iGEM Laboratory

Persons involved: Harry Fang, Annie Xian

Goals: Construction of PSB3K3-LuxR-sfGFP plasmid

Experiment steps and results:

-PCR amplification of PSB3K3-LuxR-sfGFP plasmid

-Gel Electrophoresis and extraction of PCR products

-Successful PCR amplification

-Gibson assembly of PSB3K3-LuxR-sfGFP plasmid (8 µl system, 30min at 50 degrees)

Date:20190330 Location: Haidian Experimental Middle School iGEM Laboratory

Persons involved: Harry Fang, Annie Xian

Goals: Construction of PSB3K3-LuxR-AMP plasmid

Experiment steps and results:

-PCR amplification of PSB3K3-LuxR-AMP plasmid

-Gel Electrophoresis and extraction of PCR Products

-Failed PCR amplification

Date:20190331 Location: Haidian Experimental Middle School iGEM Laboratory

Persons involved: Harry Fang, Annie Xian

Goals: Construction of PSB3K3-LuxR-AMP plasmid

Experiment steps and results:

-PCR amplification of PSB3K3-LuxR-AMP plasmid

-Gel Electrophoresis and extraction for PSB3K3-LuxR -AMP

-Successful PCR amplification

-Gibson assembly of the PSB1K3-LuxR-AMP plasmid (8 µl system, 30min at 50 degrees)

-Transfection of the plasmid into E. Coli competent cell culture

Date:20190420 Location: Haidian Experimental Middle School iGEM Laboratory

Persons involved: Harry Fang, Annie Xian, Peter Qi

Goals: Construction of PSB3K3-LuxR-AMP plasmid

Experiment steps and results:

-PCR Products prepared

-Gibson assembly of PSB3K3-LuxR-AMP plasmid (8 µl system, 30min at 50 degrees)

-Transfection of PSB3K3-LuxR-AMP plasmid into E. Coli competent cell culture

Date:20190421 Location: Haidian Experimental Middle School iGEM Laboratory

Persons involved: Harry Fang, Annie Xian, Peter Qi

Goals:

-Sequencing of successfully grown bacterial colonies

-Construction of PSB3K3-promoter plasmid

Experiment steps and results:

-PCR products prepared

-Gibson Assembly of PSB3K3-promoter plasmid (8 µl system, 30min at 50 degrees)

-Transfection of the constructed plasmid into E. Coli competent cell culture

-Picked up four positive bacterial colonies for sequencing at BGI

Date:20190512 Location: Haidian Experimental Middle School iGEM Laboratory

Persons involved: Rouxuan Yu, Annie Xian, Peter Qi

Goals: Construction of PSB3K3-LuxR-AiiA, PSB3K3-LuxR-LacZ, PSB3K3-LuxR-sfGFP plasmids

Experiment steps and results:

-PCR amplification of PSB3K3-LuxR-AiiA, PSB3K3-LuxR-Lacz, and PSB3K3-LuxR-sfGFP plasmids

-Gel electrophoresis and extraction of plasmids

-Successful amplification of all plasmids

Date:20190713 Location: Haidian Experimental Middle School iGEM Laboratory

Persons involved: Ian Liu, Annie Xian

Goals: Construction of PSB3K3-CarR-AMP, PSB3K3-CarR-LacZ, PSB3K3-CarR-sfGFP, PSB3K3-CarR-AiiA plasmids

Experiment steps and results:

-PCR amplification of all sequences

-Gel Electrophoresis and extraction of all plasmids

-Successful amplification of PSB3K3-CarR-sfGFP plasmid

-Failed amplification of PSB3K3-CarR-AMP, PSB3K3-CarR-LacZ, PSB3K3-CarR-AiiA plasmids

-Gibson assembly of PSB3K3-CarR-sfGFP plasmid(8 µl system, 30min at 50 degrees)

-Transfection of PSB3K3-CarR-sfGFP plasmid into E. Coli.

-No growth of E. Coli colonies

Date:20190714 Location: Haidian Experimental Middle School iGEM Laboratory

Persons involved: Ian Liu, Annie Xian

Goals: Construction of plasmid PSB3K3-CarR-AMP plasmid

Experiment steps and results:

-Restarting experimental progress from July 15th after discussion regarding our previous failures

Date:20190715 Location: Haidian Experimental Middle School iGEM Laboratory

Persons involved: Ian Liu, Annie Xian

Goals: Construction of PSB3K3-CarR-AMP, PSB3K3-CarR-LacZ, PSB3K3-CarR-sfGFP, PSB3K3-CarR-AiiA plasmids

Experiment steps and results:

-PCR amplification of all plasmids

-Gel Electrophoresis and extraction of all plasmids

-Failed PCR amplification of all plasmids

-Plasmid backbone too long, redesign of PCR primers

Date:20190716 Location: Haidian Experimental Middle School iGEM Laboratory

Persons involved: Ian Liu, Harry Fang

Goals: Construction of PSB3K3-CarR-AMP, PSB3K3-CarR-LacZ, PSB3K3-CarR-sfGFP, PSB3K3-CarR-AiiA

Experiment steps and results:

-PCR amplification of all plasmids

-Gel Electrophoresis and extraction of all plasmids

-Failed PCR amplification of all plasmids

-Redesign of primers

Date:20190718 Location: Haidian Experimental Middle School iGEM Laboratory

Persons involved: Harry Fang, Ian Liu, Annie Xian

Goals: Construction of PSB3K3-CarR-AMP, PSB3K3-CarR-LacZ plasmids

Experiment steps and results:

-PCR amplification of both plasmids \*(Each plasmid amplified from two different ends marked as N1 and N2, N1 being the kana antibiotic resistance gene)

-Gel Electrophoresis and extraction of both plasmids

-Both plasmids amplified successfully

-Gibson assembly of both plasmids (8 µl system, 30min at 50 degrees)

-Transfection of both plasmids into E. Coli competent cell culture

Date:20190719 Location: Haidian Experimental Middle School iGEM Laboratory

Persons involved: Ian Liu, Annie Xian

Goals: Construction of PSB3K3-CarR-AMP, PSB3K3-CarR-LacZ plasmid

Experiment steps and results:

-Successful E. Coli growth for both plasmids

-PCR amplification of E. Coli colonies

-Amplified colonies sent away for sequencing at BGI

-Correct sequence for both plasmids

Date:20190722 Location: Haidian Experimental Middle School iGEM Laboratory

Persons involved: Harry Fang, Annie Xian

Goals: Construction of PSB3K3-CarR-sfGFP, PSB3K3-CarR-AiiA plasmids

Experiment steps and results:

-PCR amplification of both plasmids \*(Each plasmid amplified from two different ends marked as N1 and N2, N1 being the kana antibiotic resistance gene)

-Gel Electrophoresis and extraction of both plasmids

-Successful amplification of both plasmids

-Gibson assembly of both plasmids (8 µl system, 30min at 50 degrees)

-Transfection of both plasmids into E. Coli competent cell culture

-No growth of E. Coli colonies

Date:20190724 Location: Haidian Experimental Middle School iGEM Laboratory

Persons involved: Annie Xian, Harry Fang

Goals: Construction of PSB3K3-CarR-sfGFP, PSB3K3-CarR-AiiA plasmids

Experiment steps and results:

-PCR amplification of both plasmids \*(Each plasmid amplified from two different ends marked as N1 and N2, N1 being the kana antibiotic resistance gene)

-Gel Electrophoresis and extraction of both plasmids

-Successful amplification of both plasmids

-Gibson assembly of both plasmids (8 µl system, 30min at 50 degrees)

-Transfection of both plasmids into E. Coli competent cell culture

-No growth of E. Coli colonies

Date:20190725 Location: Haidian Experimental Middle School iGEM Laboratory

Persons involved: Annie Xian, Harry Fang

Goals: Construction of PSB3K3-CarR-sfGFP, PSB3K3-CarR-AiiA plasmids

Experiment steps and results:

-Reconfiguration of petri dishes used for competent cell culture growth

-PCR amplification of both plasmids \*(Each plasmid amplified from two different ends marked as N1 and N2, N1 being the kana antibiotic resistance gene)

-Gel Electrophoresis and extraction of both plasmids

-Successful amplification of both plasmids

-Gibson assembly of both plasmids (8 µl system, 30min at 50 degrees)

-Transfection of both plasmids into E. Coli competent cell culture

-No growth of E. Coli colonies

-Used a competent E. Coli cell culture to test for cell death

-Failed experiment likely due to death of competent cells used for transfection

Date:20190726 Location: Haidian Experimental Middle School iGEM Laboratory

Persons involved: Peter Qi, Harry Fang

Goals: Construction of PSB3K3-CarR-sfGFP, PSB3K3-CarR-AiiA plasmids, Cell-Free synthesis of previously successfully constructed plasmids

Experiment steps and results:

-No E. Coli colony growth even when competent E. Coli cells are active

-Extraction of previously constructed and sequenced plasmids (PSB3K3-LuxR-AiiA#1, PSB3K3-LuxR-sfGFP#1, PSB3K3-LuxR-sfGFP#4, PSB3K3-LuxR-AMP#1, PSB3K3-LuxR-AMP#4) for Cell-Free synthesis

-Failed construction for both plasmids led us to buy new competent transfection cells from another source for future experiments

Date:20190727 Location: Haidian Experimental Middle School iGEM Laboratory

Persons involved: Peter Qi

Goals: Construction of PSB3K3-CarR-sfGFP, PSB3K3-CarR-AiiA plasmids

Experiment steps and results:

-PCR amplification of both plasmids \*(Each plasmid amplified from two different ends marked as N1 and N2, N1 being the kana antibiotic resistance gene)

-Gel Electrophoresis and extraction of both plasmids

-Successful amplification of both plasmids

-Gibson assembly of both plasmids (8 µl system, 30min at 50 degrees)

-Transfection of both plasmids into E. Coli competent cell culture

-Successful growth of E. Coli colonies

Date:20190728 Location: Haidian Experimental Middle School iGEM Laboratory

Persons involved: Peter Qi

Goals: Construction of PSB3K3-CarR-sfGFP, PSB3K3-CarR-AiiA plasmids, preparation of PSB3K3-LuxR-AiiA, PSB3K3-LuR-sfGFP plasmids for Cell-Free synthesis

Experiment steps and results:

-PCR amplification of E. Coli colonies for both plasmids

-Amplified plasmids sent to BGI for sequencing

-Transfer of previously constructed PSB3K3-LuxR-AiiA, PSB3K3-LuxR-sfGFP plasmids into LB solution for overnight incubation

Date:20190729 Location: Haidian Experimental Middle School iGEM Laboratory

Persons involved: Peter Qi

Goals: Extraction of incubated PSB3K3-LuxR-AiiA, PSB3K3-LuxR-sfGFP plasmids

Experiment steps and results:

-Both plasmids successfully extracted from E. Coli colonies

Date:20190730 Location: Haidian Experimental Middle School iGEM Laboratory

Persons involved: Peter Qi

Goals: Demonstration of Cell-Free synthesis and incubation of previously constructed plasmids

Experiment steps and results:

-PSB3K3-LuxR-AiiA#1, PSB3K3-LuxR-sfGFP#1, PSB3K3-LuxR-sfGFP#4, PSB3K3-LuxR-AMP#1, PSB3K3-LuxR-AMP#4 Plasmids transferred into LB solution for overnight incubation

Date:20190731 Location: Haidian Experimental Middle School iGEM Laboratory

Persons involved: Peter Qi, Harry Fang, Linda Liu, Hedy Zhu

Goals: Extraction and cell-free synthesis of PSB3K3-LuxR-AiiA#1, PSB3K3-LuxR-sfGFP#1, PSB3K3-LuxR-sfGFP#4, PSB3K3-LuxR-AMP#1, PSB3K3-LuxR-AMP#4 plasmids

Experiment steps and results:

-Successful extraction of all incubated plasmids

-Other plasmids stored in – 20-degree Celsius fridge as spare parts

-Cell-Free synthesis of extracted plasmids

-Failed synthesis

-Re-extraction of incubated plasmids successful

-Second attempt of Cell-Free synthesis failed

Experiment Protocol:

1. Preparation of liquid LB solution for competent cell culture 复苏 and plasmid extraction

|  |  |  |
| --- | --- | --- |
| Ingredients | 100ml | 250ml |
| NaCl | 1g | 2.5g |
| Tryptone | 1g | 2.5g |
| Yeast | 0.5g | 1.25g |

1. Preparation of solid LB solution for E. Coli competent cell culture petri dishes

|  |  |  |
| --- | --- | --- |
| Ingredients | 100ml | 250ml |
| NaCl | 1g | 2.5g |
| Tryptone | 1g | 2.5g |
| Yeast | 0.5g | 1.25g |
| Agarose | 1.5g | 3.75g |
| Kana Antibiotic | 100 µl | 250 µl |

1. 灭菌
2. Preparation of agarose gel
   1. Fold a weighing paper
   2. Place the folded weighing paper on an electronic balance, press ‘peeling’ to zero the balance
   3. Weigh the corresponded mass of agarose into the conical flask
   4. Pour respective volume of TAE into the conical flask
   5. Heat the solution with microwave for 3 minutes
   6. Remove the conical flask from the microwave for inspection. If the agarose is completely dissolved, proceed to step g. If the agarose is not completely dissolved, repeat step e.
   7. After cooling, add SybrSafe®(1/10000 v/v solution of the mixture) to the solution
   8. Set up the gel mould
   9. Gently pour the gel inside the mould, making sure the liquid is evenly spread
3. Polymerase Chain Reaction
   1. Set up a PCR system (Always add the Polymerase last in case of mistake)
   2. Place in PCR machine and set the program as needed
   3. 30min before PCR ends, make agarose gel for DNA recycling
   4. Add 6x Loading Buffer into each system
   5. The first lane of the left-hand side should always be the Marker (Trans2K DNA Marker)

PCR System (50 µl)

|  |  |
| --- | --- |
| Ingredients | Volume |
| 5\*Prime Star Buffer | 10 µl |
| dNTP | 4 µl |
| Forward Primer | 1.5 µl |
| Reverse primer | 1.5 µl |
| DNA template | 1 µl |
| Prime Star Polymerase | 1 µl |
| ddH2O | 31 µl |

PCR Program

|  |  |  |
| --- | --- | --- |
| Pre-Denaturing | 98℃, 2 min | 1 cycle |
| Denaturing | 98℃, 15s | 35 cycles |
| Annealing | 55℃, 15s |
| Extending | 68℃, 1kb/min |
| Further Extending | 68℃, 5min | 1 cycle |

1. Bacterial Colony Polymerase Chain Reaction
   1. Set up a big PCR system without primers
   2. Pick a single colony with gun tip and emerge it underneath the surface of the system. Blend multiple times
   3. Pipette 1µL of PCR system on the blocks already drown on the replicate plate
   4. Distribute the big system into 28µL per PCR tube
   5. Add corresponded primers (Primer F & Primer R), 1µL each to the tubes
   6. Centrifuge shortly with the microcentrifuge
   7. Vortex blending
   8. Place in the PCR machine and set the program as needed (See charts below for further details
   9. 30min before PCR ends, make agarose gel for DNA recycle (see step 6 for further details)
2. Gel Electrophoresis
   1. Emerge the gel in the TAE buffer, making sure the liquid covers all the wells
   2. Make sure the wells are placed toward the cathode
   3. Pipette 20% volume of 6x Loading buffer into the samples, blend with vortex
   4. Always put a marker in the leftmost lane
   5. Add sample that is well mixed with loading buffer into the gel, record the order on the lab note
   6. Electrophoresis should last for 30min, 150v.
   7. If Marker appears too short, run the gel for an additional 30 minutes with close monitoring
   8. After electrophoresis, obtain an image with the gel inspection machine and smart phone.
3. Recycling of PCR products
   1. Prepare a 1.5ml tube (Marked with the amplified sequence name)
   2. Placed the excised gel fragment in a 1.5ml Eppendorf tube
   3. Compress the gel on the dip of the tube using a microcentrifuge. Estimate gel volume
   4. Pipette equal volume of Buffer PN into the tube, mix with vortex, and place the tube inside a 50˚C metal bath until the gel fragment is fully dissolved
   5. Balance a CA2 spin column with 500µL Buffer BL during the time of gel dissolving 8. Centrifuge the spin column for 1min at 12000rpm, discard the flow-through
   6. Apply the dissolved gel solution into the CA2 spin column, letting stand for 2min and centrifuging at 12000rpm for 1min
   7. Collect all the flow-through back to the CA2 spin column, centrifuge at 12000rpm for 1min
   8. Discard the flow-through and pipette 600µL of Buffer PW to wash the CA2 column. Let stand for 2min then centrifuge at 12000rpm for 1min, discard the flowthrough
   9. Pipette again 600µL Buffer PW, let stand for 2min, 12000rpm for 1min, and discard the flow-through
   10. Put back the CA2 spin column, centrifuge 12000rpm for 3min without adding anything to get rid of the buffer.
   11. Apply the CA2 spin column to a new 1.5ml tube (already labeled with code on the lid, plasmid name on the body) and put inside a metal bath with lid open at 50˚C for 5min
   12. Pipette 30µL of buffer EB to the silicon film of the CA2 spin column, let stand in a 50˚C metal bath with lid closed
   13. Obtain DNA concentration with a microplate reader, note it on the body of the tube(under the name of plasmid) and date of extraction underneath that. Update into the strain list
4. Restriction Enzyme Reaction
5. Use Nanodrop to test for the concentration of the recycled PCR products
6. Obtain 1-2 µl of target fragment
7. Prepare the reaction system according to the table below
8. Incubate the system at 37℃ for three hours
9. Gel Electrophoresis and recycling of the target fragment (See steps 6 and 7 for details)

Restriction Enzyme System (50µl)

|  |  |
| --- | --- |
| Ingredients | Volume |
| NEB Enzyme I | 1µl |
| NEB Enzyme II | 1µl |
| Target Fragment | 1-2µg |
| 10\*Cutsmart Buffer | 5µl |
| ddH2O | 41-42µl |

1. Gibson Assembly
   1. Using 5µL smart mix to construct a Gibson assembly system
   2. Set PCR program as: 50˚C 20min҅12˚C forever
   3. Blend Gibson system with 100µL of competent cells, ice bath over 30 min (Allow thorough contact of DNA and cells, enabling DNA to enter the cell wall)
   4. Heat shock at 42˚C for 90s (Cells swell at high temperature, allow thorough contact of the cell membrane and cell wall, enabling DNA to pass through the cell membrane into the cytoplasm)
   5. Ice bath for 5min(Cell shrinks at low temperature, dragging DNA on the wall into the cell)
   6. Pipette 200µL of all nutrient broth into the tube(operate inside the bench), shake the bacteria in a shaker at 37˚C for 1h30min
   7. Spread the bacteria on plates. Observe after 16h~20h
2. Preparation of petri dishes for competent cell culture
3. Solid LB solution prepared
4. Carefully pour the solution into clean petri dishes in ventilated Cell Culture Hood
5. Allow the solution to dry over 30 minutes
6. Seal the petri dishes with tape
7. Store in 4˚C fridge, upside down to prevent accumulation of moisture
8. E. Coli Competent Cell Culture Transfection
9. Place 50µl of Trans5α competent cells culture onto ice
10. Add the reacted fragment-backbone mixture solution into the competent cell culture
11. Place the mixed solution onto ice for 30 minutes
12. Stimulate the solution in 42˚C water bath for 60 seconds
13. Place the solution onto ice for two minutes
14. Add 1ml of liquid LB without antibiotics in ventilated Cell-Culture Hood
15. Proceed with step 12
16. Incubation
17. Prepare and label an empty 15 ml test tube
18. Pipette 400µl of liquid LB solution into the test tube
19. Pipette the bacteria solution into the test tube with liquid LB
20. Place the test tube into 37˚C incubation for 45 minutes
21. Plasmid Extraction (Omega E.Z.N.A. Plasmid DNA Mini Kid I)
22. Isolate a single colony from a freshly streaked selective plate, and inoculate a culture of 1- 5 mL LB medium containing the appropriate selective antibiotic. Incubate for ~12-16 hr at 37°C with vigorous shaking (~ 300 rpm). Use a 10-20 mL culture tube or a flask with a volume of at least 4 times the volume of the culture. It is strongly recommended that an endA negative strain of *E. coli* be used for routine plasmid isolation. Examples of such strains include DH5a® and JM109®.
23. Centrifuge at 10,000 x *g* for 1 minute at room temperature.
24. Decant or aspirate and discard the culture media.
25. Add 250 μL Solution I/RNase A. Vortex or pipet up and down to mix thoroughly. Complete resuspension of cell pellet is vital for obtaining good yields.

**Note:** RNase A must be added to Solution I before use. Please see the instructions in the Preparing Reagents section on Page 6.

1. Transfer suspension into a new 1.5 mL microcentrifuge tube.
2. Add 250 μL Solution II. Invert and gently rotate the tube several times to obtain a clear lysate. A 2-3 minute incubation may be necessary.

**Note:** Avoid vigorous mixing as this will shear chromosomal DNA and lower plasmid purity. Do not allow the lysis reaction to proceed more than 5 minutes. Store Solution II tightly capped when not in use to avoid acidification from CO2 in the air.

1. Add 350 μL Solution III. Immediately invert several times until a flocculent white precipitate forms. **Note:** It is vital that the solution is mixed thoroughly and immediately after the addition of Solution III to avoid localized precipitation.
2. Centrifuge at maximum speed (≥13,000 x *g*) for 10 minutes. A compact white pellet will form. Promptly proceed to the next step.
3. Prepare the vacuum manifold according to manufacturer’s instructions.
4. Connect the HiBind® DNA Mini Column to the vacuum manifold.
5. Transfer the cleared supernatant from Step 8 by CAREFULLY aspirating it into the HiBind® DNA Mini Column. Be careful not to disturb the pellet and that no cellular debris is transferred to the HiBind® DNA Mini Column.
6. Turn on the vacuum source to draw the sample through the column.
7. Turn off the vacuum.
8. Add 500 μL HBC Buffer.

**Note:** HBC Buffer must be diluted with 100% isopropanol before use. Please see Page 6 for instructions.

Turn on the vacuum source to draw the buffer through the column.

Turn off the vacuum.

1. Add 700 μL DNA Wash Buffer.

**Note:** DNA Wash Buffer must be diluted with 100% ethanol prior to use. Please see Page 6 for instructions.

1. Turn on the vacuum source to draw the buffer through the column.
2. Turn off the vacuum.
3. Repeat Steps 17-19 for a second DNA Wash Buffer wash step.
4. Transfer the HiBind® DNA Mini Column to a 2 mL Collection Tube.
5. Centrifuge the empty HiBind® DNA Mini Column for 2 minutes at maximum speed to dry the column matrix.

**Note:** It is important to dry the HiBind® DNA Mini Column matrix before elution. Residual ethanol may interfere with downstream applications.

1. Transfer the HiBind® DNA Mini Column to a clean 1.5 mL microcentrifuge tube.
2. Add 30-100 μL Elution Buffer or sterile deionized water directly to the center of the column membrane.

**Note:** The efficiency of eluting DNA from the HiBind® DNA Mini Column is dependent on pH. If using sterile deionized water, make sure that the pH is around 8.5.

1. Let sit at room temperature for 1 minute.
2. Centrifuge at maximum speed for 1 minute. **Note:** This represents approximately 70% of bound DNA. An optional second elution will yield any residual DNA, though at a lower concentration.
3. Store DNA at -20°C.
4. Cell-Free Synthesis
5. Preparation of Cell-Free system
6. Incubation of prepared system at 37℃ for 3-6 hours

Cell-Free System

|  |  |  |
| --- | --- | --- |
| Ingredients | Volume | |
| 10\*NaCl | 2µl | 12µl |
| PEP | 0.8µl | 4.8µl |
| NTP | 0.8µl | 4.8µl |
| 19AA | 0.8µl | 4.8µl |
| Mg | 0.4µl | 2.4µl |
| Cell-Extract | 6µl | 36µl |
| PEG 8000 | 2.5µl | 15µl |
| T7 RNAP | 0.1µl | 0.6µl |
| ddH2O | 3.6µl | 21.6µl |
| Template (300 ng) | 3µl | µl |