Chapter 26

Designing and Assembling Plasmids for the Construction of *Escherichia coli* Biosensor for *Vibrio cholerae* Detection

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Abstract

In the process of constructing and characterizing the whole cell biosensor for *Vibrio cholerae* detection, two main techniques have been employed—DNA assembly using the Gibson isothermal assembly reaction was used for the assembly of the PCRed plasmid fragments (DNA parts), and microplate fluorescence readings were used for bacterial strain characterization. The general workflow can be summed up as: the in silico designed DNA fragments were assembled by isothermal assembly to be later transformed into *Escherichia coli* that, in turn, was characterized using the microplate reader. As fine-tuning of the sensor design was required, the process was repeated iteratively until the final strain was created with desired characteristics. This chapter describes in detail this workflow for different constructs which finally led to the creation of the first whole cell biosensor in *E. coli* for *V. cholerae* detection.

Key words *Vibrio cholerae*, CRISPRi, Synthetic biology, Biosensing, Infectious disease, Quorum sensing

1 Introduction

Vibrio cholerae is the causative agent of the cholerae disease. The main symptom of cholera is a profound diarrhoea that leads to severe dehydration and, finally, death of the infected person. Cholera is still present in many areas of the world in both endemic and epidemic fashions [1, 2]. Currently, we lack effective means of therapy and prevention of cholera. The state-of-the-art therapy, oral rehydration therapy, prevents death of the patients, but does not stop the spread of the bacteria since it is aimed only at alleviating the symptoms [3]. Detection of cholera is done either by slow and cheap cultivation methods or fast and expensive molecular based methods like PCR [4]. Prevention done via vaccines is also ailing due to their low efficacy [5]. To help with improving the *V. cholerae* detection capabilities, a novel *E. coli* based biosensor has been created using synthetic biology tools [6].

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Synthetic biology uses engineering principles to create novel features in modified organisms [7]. One of the main paradigms of synthetic biology is the use of basic genetic parts which constitute the complex genetic circuits [8]. This chapter describes how genetic circuits for the *V. cholerae* detection were created. Detection of the bacteria itself is done by repurposing the native *V. cholerae* quorum sensing mechanism which it uses for the coordination of infection [9]. This quorum sensing system in *V. cholerae* is highly parallelized and uses different protein sensors for detecting a set of chemical compounds [9]. One of these compounds, CAI-1 (cholera autoinducer 1), is known to be highly specific toward *V. cholerae* [10]. CAI-1 is detected by a two-component sensor protein CqsS. CqsS in turn controls phosphorylation of LuxU, a phosphorelay protein. Finally, LuxU controls phosphorylation of LuxO, which is a transcription factor for promoters for small quorum sensing regulated RNAs called Qrrs. When CAI-1 is in low concentration in the environment (low *V. cholerae* density), CqsS phosphorylates LuxO via LuxU, which starts expression of the Qrrs. When the CAI-1 concentration is high (high *V. cholerae* density), the flow of phosphate groups is reversed and Qrrs are not expressed anymore. As a result, the system is OFF when CAI-1 is high, a situation opposite to the desired one if one considers that the reporter protein (in this case GFP) should be produced when the CAI-1 level is high. To solve this problem, a CRISPRi-based inverter was used in the circuit [11]. CRISPRi was chosen since it is easily modifiable to achieve desired characteristics (e.g., level of repression, sensitivity, output promoter strength).

The whole system is divided into three separate modules (i.e., sensing, inverting, and actuator). The modules were designed, assembled, and characterized separately before assembling them into the final system with the aid of a computer model [6]. The computer model, which described the phosphorylation cascade and the inverter, was developed to help in determining the parts of the system, which were critical to its performance. The full system is illustrated in Fig. 1. All the plasmid maps, sequences, and other important theoretical data are available in the supporting information for work by Holowko et al. [6].

2 Materials

2.1 DNA Design

1. Benchling design software (available at www.benchling.com).

2.2 General Isothermal Assembly and Transformation

1. PCR primers designed using Benchling.
2. Q5 High Fidelity Polymerase kit from NEB or equivalent.
3. dNTP solution.
4. Thermocycler.

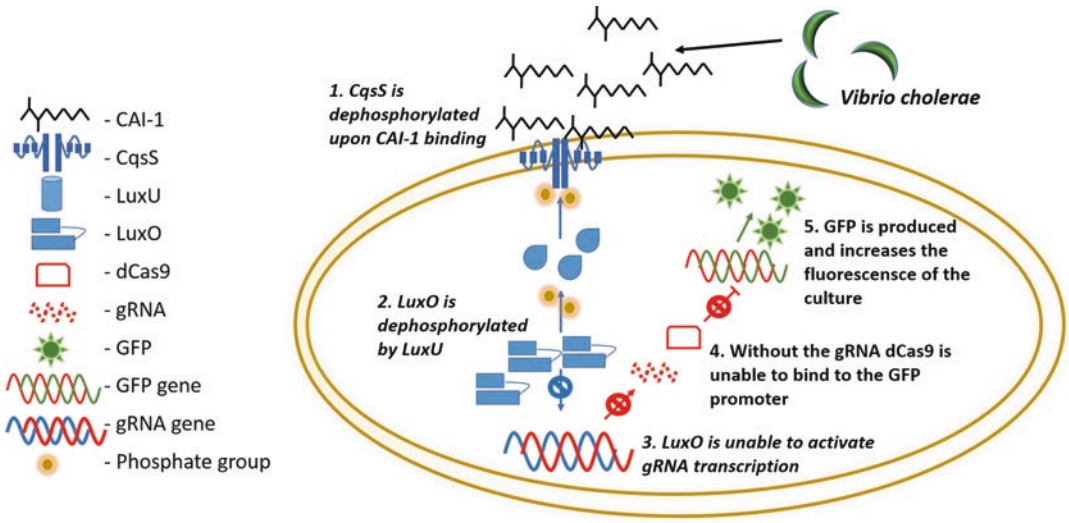

Fig. 1 Overview of the final device in the modified *E. coli*

5. Plastic PCR tubes.
6. Gel electrophoresis setup.
7. 1× TAE (40 mM Tris base, 40 mM acetic acid, 1 mM EDTA).
8. 1% agarose gel in TAE.
9. SYBR Safe Stain or equivalent.
10. 1 kb DNA Generuler Ladder from Thermofisher Scientific or equivalent.
11. DNA Gel Loading Dye 6× from Thermofisher Scientific or equivalent.
12. UV illuminator.
13. Scalpel.
14. Qiagen gel extraction kit or equivalent.
15. Nanodrop 2000.
16. NEBuilder Master Mix from NEB.
17. Thermoblock.
18. 10-beta Competent *E. coli* (High Efficiency) from NEB.
19. LB broth, sterile.
20. Kanamycin stock solution ×1000 (50 mg/mL, in water).
21. Ampicillin stock solution ×1000 (100 mg/mL, in water).
22. Chloramphenicol stock solution ×1000 (25 mg/mL, in ethanol).
23. Petri dish.
24. LB agar, sterile.

25. L-shaped spreader.
26. Shaking incubator.
27. Stationary incubator.
28. 50 mL centrifuge tube.
29. Chemically competent *E. coli* MG1655.
30. Qiagen Miniprep spin kit or equivalent.
31. 1.5 mL plastic tubes.
32. Benchtop centrifuge for 1.5 mL plastic tubes.
33. Glycerol, sterile.
34. −80 °C refrigerator.

2.3 General Characterization

1. LB Broth, sterile.
2. Glycerol stock of the tested *Escherichia coli* strain.
3. 50-mL centrifuge tube.
4. Shaking incubator.
5. Spectrophotometer with capability to take absorbance measurements.
6. Microplate reader with incubation, shaking, and fluorescence/absorbance reading capabilities.
7. 96-well transparent microplate with flat-bottom wells.
8. Inducer stock solutions.
9. Antibiotic stock solutions.

2.4 Actuator Module Characterization

1. Source plasmids or synthetic sequences for plasmids A1-A4 construction.
2. Chemically competent *E. coli* MG1655.
3. Arabinose stock solution 15% w/v in water.
4. Ampicillin stock solution ×1000 (100 mg/mL, in water).

2.5 Inverter Module Characterization

1. Source plasmids or synthetic sequences for plasmids C1–C12 construction.
2. Chemically competent *E. coli* MG1655.
3. Isopropyl β-D-1-thiogalactopyranoside (IPTG) stock solution 1 M in water.
4. Anhydrotetracycline (ATc) stock solution 215 μM in 1:1 water:ethanol.
5. Kanamycin stock solution ×1000 (50 mg/mL, in water).
6. Chloramphenicol stock solution ×1000 (25 mg/mL, in ethanol).

2.6 Sensor Module Characterization

1. Source plasmids or synthetic sequences for plasmids D2 with variants and D3 construction.
2. Chemically competent *E. coli* MG1655.
3. Kanamycin stock solution ×1000 (50 mg/mL, in water).
4. Chloramphenicol stock solution ×1000 (25 mg/mL, in ethanol).

2.7 Final System Characterization

1. Source plasmids or synthetic sequences for plasmids C13 with variants and D2 construction.
2. Chemically competent *E. coli* MG1655.
3. *E. coli* MG1655 from ATCC.
4. *V. cholerae* strain A1522.
5. LB Broth, sterile.
6. 50-mL centrifuge tube.
7. Shaking incubator set at 37 °C.
8. 10-mL syringe.
9. 0.2 μm microbial syringe filter.
10. Benchtop centrifuge for 50 mL centrifuge tubes.
11. Spectrophotometer with capability to take absorbance measurements.
12. Microplate reader with incubation, shaking, and fluorescence/absorbance reading capabilities.
13. 96-well transparent microplate with flat-bottom wells.
14. Isopropyl β-D-1-thiogalactopyranoside (IPTG) stock solution 1 M in water.
15. Kanamycin stock solution ×1000 (50 mg/mL, in water).
16. Chloramphenicol stock solution ×1000 (25 mg/mL, in ethanol).

3 Methods

3.1 DNA Design

This method explains how to design the isothermal DNA assembly using the Benchling designer (*see* **Note 1**). The advantages of this solution are the ease of use of the built-in assembly wizard, accurate primer design, high chance of avoiding mispriming events, and high level of possible customization.

1. Go to www.benchling.com and create an account; all the subsequent steps are done in Benchling (*see* **Note 2**).
2. Upload all the required sequences like backbones and parts as Genbank, FASTA, or SBOL files (*see* **Note 3**).

3. Annotate all your sequences properly; especially all promoter, RBS, CDS, terminator, and plasmid origin sequences need to be annotated.
4. gRNA sequences for CRISPRi should be designed using the CRISPR design tool in Benchling Follow Benchling documentation for the design.
5. Run the Assembly Wizard and choose Gibson assembly method.
6. Select your backbone and insert sequences
7. Modify the sequence as needed—add spacers and reverse sequences (*see* **Notes 4** and **5**).
8. Click on Assemble.
9. Check if the designed sequence is in the right order and is exactly what you needed.
10. Check the primers length and mispriming in the Assembly tab.
11. Accept the assembly in the Assembly tab.

3.2 General Isothermal Assembly and Transformation

This subsection explains how to create, assemble, and transform parts designed using the Benchling software. It starts with PCRing the parts, which are later isothermally assembled using NEBuilder and finally transformed into 10-beta and MG1655 cells.

1. Order primers for the planned construction (as per Benchling generated protocol) (*see* **Note 6**).
2. Perform PCR following the polymerase manufacturer manual; 50 μL volume.
3. Prepare 1% agarose gel with the required dye in TAE.
4. Run the PCR generated assembly parts (whole PCR volume; mixed with loading dye) with the ladder in the gel (130 V until the gel is around 60% developed).
5. Visualize the gel with UV illuminator and check if the sizes of the parts are correct (*see* **Note 7**).
6. Excise the bands using a scalpel.
7. Extract the DNA using the gel extraction kit following the manufacturer's manual.
8. Quantify the DNA concentrations of individual parts using the Nanodrop.
9. Mix the parts in ratios recommended by NEBuilder manual.
10. Add the NEBuilder Master Mix to the parts mix to 20 μL volume.
11. Heat the mixture to 50 °C for 15–30 min (*see* **Note 8**).
12. Transform the *E. coli* 10-beta following the manufacturer's manual (use 2 μL of the assembled plasmid) (*see* **Notes 9** and **10**).

13. Plate the transformed cells on a Petri dish with corresponding antibiotic.
14. Incubate the plate overnight in 37 °C.
15. Choose 4 colonies from the plate and incubate them overnight in 37 °C in 6 mL LB broth with corresponding antibiotic.
16. Extract plasmids from the overnight cultures using Miniprep kit following the manufacturer's manual.
17. Sequence the plasmids to find the correct clones.
18. Use the correct plasmid to transform *E. coli* MG1655 (you can follow the same protocol as for 10-beta cells; use 1–2 μL of the assembled plasmid).
19. Plate the transformed cells on a Petri dish with corresponding antibiotic.
20. Incubate the plate overnight in 37 °C.
21. Choose 1 colony from the plate and incubate it overnight in 37 °C in 10 mL LB broth with corresponding antibiotic.
22. Prepare a 50:50 mixture of LB with transformed *E. coli*: Glycerol and keep it in −80 °C as your glycerol stock.

3.3 General Characterization

This method describes how the characterization of circuits created for this work was performed. The general protocol is the same for all the circuits, with differences listed in the given subsections.

1. Prepare 5 mL of LB supplemented with antibiotic matching resistance of the tested strain in a 50 mL centrifuge tube.
2. Inoculate the prepared LB broth with the tested strain using inoculation loop from the glycerol stock tube.
3. Incubate the culture overnight in the shaking incubator at 37 °C.
4. Prepare 1 mL of LB broth supplemented with corresponding antibiotic in 50 mL tube per each tested concentration of inducer. For example, if one is testing five different concentrations of arabinose 5 mL of culture needs to be prepared.
5. Inoculate the freshly prepared LB with the overnight culture in a ratio of 100:1.
6. Incubate the prepared culture in a shaking incubator set at 37 °C for 2–3 h until the culture hits OD_{600} of 0.1.
7. Load the microplate with the inducers into the wells in triplicates before adding the cultures. For example if the concentration of arabinose to be tested is 0.05% then you would add 1 μL of 15% stock solution to the well prior to adding the 300 μL of culture to the said well (*see* **Note 11**).
8. Add the culture to the microplate in triplicates (volume of 300 μL) to the corresponding wells with inducers. Include blank and control (no inducer) in triplicate.

9. Run the microplate in the reader with the following settings:
   (a) Time: 8 h.
   (b) Shaking: Medium to Vigorous.
   (c) Reading interval: 10 min.
   (d) Fluorescence reading at GFP wavelengths.
   (e) Absorbance reading at 600 nm.
10. Collect and process the data.

3.4 Actuator Module Characterization

This subsection explains how one should proceed with the characterization of the actuator module.

1. Design, create, and transform plasmids A1–A4 using the steps described in Subheadings 3.2 and 3.3 using Ampicillin as your selection antibiotic.
2. Run the microplate characterization according to Subheading 3.3 with the following modification:
   (a) In point 7 add the stock solution of the arabinose to the wells to achieve final concentrations of: 0, 0.000005, 0.00001, 0.0001, 0.0005, 0.001, 0.002, 0.005, 0.01, 0.02, 0.05, 0.075, and 0.125%.

3.5 Inverter Module Characterization

This subsection explains how one should proceed with the characterization of the inverter module.

1. Design, create, and transform plasmids C1–C4 and C5–C12 using the steps described in Subheadings 3.1 and 3.2 using chloramphenicol for C1–C4 and kanamycin for C5–C12 as your selection antibiotic.
2. Prepare double plasmid transformed cells with one of the C1–C4 plasmids with corresponding C5–C12 plasmids. For example if your chosen plasmid C1 has Anderson promoter BBa_J23115 [12] then your C5 and C6 plasmids should express gRNAs targeted at its −10 and −35 sites respectively. Use chloramphenicol and kanamycin as your selection markers for these cultures.
3. Run the microplate characterization according to Subheading 3.3 with the following modification:
   (a) In point 7 first add the stock solution of IPTG in a way that the final concentration of IPTG will increase in rows (final concentrations of IPTG should be: 0, 3 μM, 6 μM, 12 μM, 25 μM, 50 μM, 100 μM, and 500 μM). Do the same for ATc, but its concentration should increase in columns (final concentrations of ATc should be 0, 0.1 nM, 0.25 nM, and 0.5 nM). This way in wells A1–A3 you will

have no inducers and in wells H10–H12 you will have maximum concentration of both IPTG and ATc (500 μM and 0.5 nM) respectively.

3.6 Sensor Module Characterization

This subsection explains how one should proceed with the characterization of the sensor module.

1. Design, create, and transform plasmids D2 and its RBS variations, and D3 using the steps described in Subheadings 3.2 and 3.3 using kanamycin (for D2) and chloramphenicol (for D3) as your selection antibiotics.
2. Prepare double plasmid transformed cells with one of the six variants of D2 plasmid with D3 plasmids. Use chloramphenicol and kanamycin as your selection markers for these cultures.
3. Run the microplate characterization according to Subheading 3.3 with the following modification:
   (a) No inducers are added in point 7 as the systems are fully constitutive.

3.7 Final System Characterization

This chapter explains how one should proceed with the characterization of the fully assembled module (*see* **Notes 12** and **13**).

1. Design, create, and transform plasmids D1 and C13 using the steps described in Subheadings 3.1 and 3.2 using kanamycin (for D1) and chloramphenicol (for C13) as your selection antibiotics.
2. Prepare double plasmid transformed cells with one of the 6 variants of D2 plasmid with D3 plasmids. Use chloramphenicol and kanamycin as your selection markers for these cultures.
3. Prepare 5 mL of LB supplemented with antibiotic matching resistance of the tested strain in 50 mL centrifuge tube.
4. Two additional tubes for *V. cholerae* and wild type *E. coli* need to be prepared and cultured. Each of them with 10–20 mL of LB broth and no antibiotic.
5. Inoculate the prepared LB broths with the respective strains using inoculation loop from the glycerol stock tube.
6. Incubate the cultures overnight (12–14 h) in the shaking incubator at 37 °C.
7. The following has to be done separately for overnight cultures of *V. cholerae* and wild type *E. coli*:
   (a) Centrifuge the tube at approximately 3700 × *g* for 8 min at 4 °C.
   (b) Remove the obtained supernatant from the tube into a 10 mL syringe.

(c) Attach the 0.2 μm filter and empty the syringe through it to a fresh, sterile 50 mL tube.

8. Prepare of mixtures of supernatants with fresh LB, separately for *V. cholerae* supernatant and *E. coli* wild type supernatant. The mixtures should contain 5, 30, 50, and 90% of supernatant for a total of eight tubes. Each tube should be 10 mL in volume. Add 10 μL chloramphenicol and kanamycin stock solutions to each tube.
9. Inoculate such prepared tubes with the overnight tested *E. coli* culture in a ratio of 100:1.
10. Incubate the prepared culture in a shaking incubator set at 37 °C for 2–3 h until the culture hits OD_{600} of 0.1.
11. Load the microplate with the IPTG stock solution into the wells in triplicates before adding the cultures. The final concentrations of IPTG tested are: 0, 25 μM, 50 μM, 100 μM, 200 μM, 300 μM, or 500 μM.
12. Add the culture to the microplate in triplicates (volume of 300 μL) to the corresponding wells with inducers. Include blank and control (no inducer) in triplicate.
13. Run the microplate in the reader with the following settings:
    - Time: 8 h.
    - Shaking: Medium to Vigorous.
    - Reading interval: 10 min.
    - Fluorescence reading at GFP wavelengths.
    - Absorbance reading at 600 nm.
14. Collect and process the data.

4 Notes

1. If in doubt on how to use some kit or other materials, always use the manufacturer's manual. They often include very useful troubleshooting tips.
2. Benchling has a quite informative tutorial and documentation. You should go through them before you design any plasmids.
3. If you know given part's registry designation a very easy way to get its sequence into your Benchling repository is simply providing the said designation to Benchling using its import function.
4. If mispriming occurs you can simply try moving the primers 3–5 bp toward any end of the DNA as long as the enough homology for the isothermal assembly is maintained.

5. It is usually advantageous to introduce 20–30 bp spacers between sequences that end with a terminator to allow for easier PCRs later.
6. A very good alternative to obtaining a DNA part by PCRing it from an existing plasmid is ordering a synthetically synthesized part from one of the companies offering such services.
7. If size of any of the parts (as seen in the gel) is wrong, it is best to return to the designer software and redesign the primers by moving them 3–5 bp toward any end of the DNA as long as the enough homology for the isothermal assembly is maintained.
8. It is often beneficial to run your isothermal assembly longer, up to 60 min, if you have more parts than 3 or they are long (above 3000 bp).
9. It is beneficial to first transform into 10-beta cells as they have much higher transformation efficiency and only then transforming the target strain (wild type with high expression rates) with the purified plasmid.
10. If your transformation efficiency is low try adding more volume of the assembly mixture to the transformed cells, instead of 2 μL try 5 μL or 10 μL.
11. You should dilute inducer stock solutions for easier application to the wells. The inducer stock solution added to the wells should not exceed 5 μL, if your calculations show that adding more than that is required, just divide that amount by 10 and use one fold less diluted stock solution.
12. It is best to inoculate your LB for experiments with glycerol stock cultures.
13. Since the microplate has only 96 wells and you need more than 160 to run the last protocol fully, it is best to divide it into two separate runs with two concentrations of supernatants each time.

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