IBEHS 2P03: Health Solutions Design Projects II

M3: Materials and Methods for a Biological System Detecting Heavy Metals and Contaminants in Drinking Water

Group 21

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Materials

Potted pothos ivy plants were obtained from a local garden store (Home Depot, Niagara Falls, Canada). *Agrobacterium tumefaciens* strain, EHA105, was obtained from Lifeasible (New York, United States). The genetic sequences for a merR codon-determining sequence (CDS) and green fluorescent protein CDS (K1513002), mercury-sensing promoter (PmerT) CDS (K346002), constitutive promoter (J23100), ribosome-binding sequence (RBS) (B0034), lead-binding CDS (I721002), two termination sequences (B0010, B0012), and the yellow chromoprotein (amilGFP) CDS (K592010) are found on the iGEM registry. The individual parts available for purchase on the iGEM registry are too short for primer attachment, prior to polymerase chain reaction amplification. Therefore, the parts will be synthesized via oligonucleotide synthesis and purchased from a custom gene synthesis site, GenScript (New Jersey, United States). Liquid mercury and lead metal powder were obtained from Lab Alley (Texas, United States). For ligation, T4 DNA ligase, 5U/µL and 10X T4 ligase buffer were used from Thermo Fisher Scientific (Ontario, Canada).

Methods

Plant Materials

Potted pothos ivy plants were grown in 16h/8h light/dark cycles, at 25°C, following protocol from Zhang et al [1]. Stem fragment samples were taken from the plants and sterilized with 15 wt% sodium hypochlorite [1]. Fragments were rinsed three times with sterile, deionized water. Fragments were cultured on Murashige and Skoog's (MS) medium for 1-2 months [2].

Assembling the Vector

Amplifying Genes of Interest

The genes of interest were amplified using polymerase chain reaction (PCR). Details regarding PCR primers, including primer melting temperature (T_m), %GC, length of the primer, and restriction overhang sites are tabulated in Table 1. The genes were synthesized together, based on the gene group's function. Parts responsible for mercury sensing, K346002, B0030, K1513002, were synthesized respectively, within one larger gene fragment (GenScript, New Jersey, United States). Parts responsible for lead sensing, J23100, B0034, I721002, B0010, and B0012, were synthesized respectively, within one larger gene fragment (GenScript, New Jersey, United States). K592010, the yellow chromoprotein, was purchased as a synthesized gene fragment from the iGEM registry. A pair of primers was designed for each composite part (Table 1).

Table 1. The genes amplified through PCR and their respective PCR conditions (primer annealing temperature, T_a is T_m- 5 ℃) primer sequences, and restriction sites.

Gene of Interest	Primer Sequences	Restriction Sites
K592010: amilGFP, yellow chromoprotein	5' ATGTCTTATTCAAAGCATGGC 3' T_M= 51.18℃, %GC = 44.82%, length=21bp 5' TTATTATTTAACCTTCAAAGGGT 3' T_M = 47.67℃, %GC = 34.48%, length=23bp	FspAI, BamHI
K346002, B0030, K1513002: PmerT promoter Ribosome-binding site, Mercury-binding CDS and GFP reporter CDS	5'TTTCATATTGCCTAACTTCGT 3' T_M = 49.54℃, %GC = 33.33%, length=21bp 5' AAGCCGCAAGAAAGCCCA 3' T_M = 56.86℃, %GC = 57.99%, length=18bp	EcoRI, Eco81I
J23100, B0034, I721002, B0010, B0012: Promoter, RBS, lead-binding CDS, termination sequence	5' CTCACGGCCTCCTCGGTG 3' T_M = 58.87℃, %GC = 70%, length=18bp 5' GATGAATGCTGAAAGGCCCA 3' T_M = 54.9℃, %GC = 53.57%, length=20bp	Eco81I, FspAI

PCR was performed using the reagents listed in Table 2 for 3 different DNA templates (Table 1). For each DNA template, the listed reagents were assembled within a microcentrifuge tube and kept on ice until the tubes were loaded into a Thermocycler. Protocol was adapted from [3]. The thermocycler conditions are listed in Table 3.

Table 2. List of reagents used in PCR for 1 aliquot of template DNA.

1 µL, 10µM forward (Table 1)
1 µL, 10µM reverse primer
22 µL nuclease free water
25 µL Taq 2x MasterMix
1 µL template DNA

Table 3. Thermocycler conditions for gene of interest amplification via PCR, replicated from where T_m is specific to one of 3 pairs of primers in Table 1, dependent on the template DNA being amplified [4].

1. T_m for 5 mins.
2. T_m for 0.5 mins.
3. T_a for 0.5 mins.
4. 72°C for 1 min.
5. Repeat 2-4 29 times.
6. 72°C for 10 min.
7. Hold at 4°C.

Purification

The amplified PCR products need to be isolated from all other components that were present in the reaction. To purify the product, we will use the PureLink PCR Purification kit which removes primers, dNTPs, enzymes, and salts. The three general steps in this process are: binding, washing, and eluting. First, add binding buffer with isopropanol to the PCR product in a spin column. Then, centrifuge the column and discard the flowthrough. Add wash buffer with ethanol into the same spin column and then centrifuge it. Discard the collection tube and place the spin column in a clean elution tube. Add elution buffer to the column and then, incubate at room temperature for 1 minute. Centrifuge the column again and now, the elution tube contains the purified product [5].

Ligation

Taking the purified PCR products, aliquot the amounts shown in Table 4 to their respective 1.5 mL microcentrifuge tube. Tubes 1and 4 contain the backbone and PCR sequences, while tubes 2 and 6 contain a cut plasmid (negative control), and tubes 3 and 7 contain an uncut plasmid (positive control).

Table 4. Amounts of reagents needed for ligation for the genes of interest and controls.

	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5	Tube 6	Tube 7
Reagent	Volume (µL)			Reagent	Volume (µL)		
Backbone DNA	10.0	10.0	10.0	Backbone DNA	10.0	10.0	10.0
K346002, B0030, K1513002: PmerT promoter (mercury-responsive), Ribosome-binding site, Mercury-binding CDS and GFP reporter CDS	4.0	0.0	0.0	amilGFP, yellow chromoprotein	2.0	0.0	0.0
10X ligase buffer	2.0	2.0	2.0	J23100, B0034, I721002, B0010, B0012: Promoter, RBS, lead-binding CDS, termination sequence	1.5	0.0	0.0
T4 DNA ligase	1.0	1.0	1.0	10X ligase buffer	2.0	2.0	2.0
Water	3.0	7.0	7.0	T4 DNA ligase	1.0	1.0	1.0
				Water	3.5	7.0	7.0

Once added, incubated for 10 minutes at room temperature.

Agrobacterium Culture

A. tumefaciens strain EHA105 was obtained from Lifeasible and the final vector was transferred using freeze-thaw Lifeasible protocol [6]. 0.01-1µg of the final ligated DNA plasmid was added to 100µL of *A. tumefaciens* cells and mixed by hand. The sample was placed on ice for 5 minutes, in liquid nitrogen for 5 minutes, water bath at 37°C, and finally, on ice for 5 minutes. The culture was grown in antibiotic-free LB medium on a shaker for 2-3 hours at 28°C. The culture was centrifuged at 6000rpm for 1 minute and resuspended in 100 µL of the supernatant. The solution was added to an LB plate containing 50ug/mL of rifampicin and cultured at 28°C for 72-90 hours, or until optical density at 600nm reached 0.8-1.0 [1].

In vitro Tests

Negative Control

When mercury was absent from the LB plate containing the plasmid with the insert, stability of cell growth and cell colour were observed. For a successful negative control, no green colour change should be observed because fluorescent GFP will not be transcribed from the gene. When

lead was absent from the LB plate containing the plasmid with the insert, stability of cell growth and cell colour were observed. For a successful negative control, no yellow colour change should be observed because fluorescent yellow chromoprotein will not be transcribed. When both metals were absent from the LB plate containing the plasmid with the insert, stability of cell growth and cell colour were observed. For a successful negative control, no fluorescent green nor yellow colour change should be observed because neither fluorescent protein should be transcribed.

Cell colonies comprised of cells containing the plasmid DNA without the insert should also be observed for stability of cell growth and cell colour. Colonies should still appear because the plasmid will contain the rifampicin resistance gene. No colour should be observed because sequences for fluorescent proteins will not be contained within the cell genome.

Positive Control

When mercury was added to the LB plate containing the plasmid with the insert, stability of cell growth and cell colour was observed. For a successful positive control, the cell colonies should turn fluorescent green, due to transcription of GFP. When lead was added to the LB plate containing the plasmid with the insert, stability of cell growth and cell colour was observed. For a successful positive control, the cell colonies should turn fluorescent yellow, due to transcription of fluorescent yellow chromoprotein. When both metals were present on the LB plate containing the plasmid with the insert, stability of cell growth and cell colour were observed. For a successful positive control, a fluorescent yellow green because both fluorescent proteins should be transcribed.

Transforming Pothos Ivy Plants

Zhang et al. protocol was followed to transform pothos ivy fragments with the modified *A. tumefaciens* culture [1]. Fragments were immersed in *A. tumefaciens* culture for 20 minutes and transferred to filter paper wetted with a liquid E medium - 100uM acetosyringone (to promote bacterial infection) mixture and cocultured for 5 days at 25°C [7]. After 5 days, the fragments were cultured in liquid E medium. Fragments were subcultured with fresh media every 3 weeks. Upon development of somatic embryos 2-3 months later, fragments were transferred to fresh media for 1 month, and following, transferred to G medium, a culture medium similar to liquid E medium, for an additional 2 months. Like liquid E medium, G medium contains 1-napthaleneacetic acid for encouraging root growth [8]. G medium does not contain thidiazuron but does contain 6-benzylaminopurine, a compound that promotes plant growth and development [10]. Finally, the plantlets were transferred to MS medium for culturing for 2 months [1].

Modelling and Data Analysis

Using Matlab Simbiology, various simulations were run to demonstrate the effectiveness of the biological circuits. mRNA species for each of the 3 synthesized sequences, two metal species for the metal inputs, mercury and lead, and the two transcribed protein species, GFP and yellow chromoprotein were defined within the model. Half lives of each protein, GFP and yellow chromoprotein, kinetic constants for transcription and translation, and degradation constants for each protein and each mRNA were defined as parameters. Reactions were defined following theory presented in [10]. Graphs were produced based on varying concentrations of mercury and lead and their respective outputs concentrations of GFP and yellow chromoprotein. The expected outputs

increased and decreased based on higher and lower contaminant concentrations, respectively, indicating that the biological circuit and reactions are working as intended, without interfering with each other. Length of run time was also varied to see how quickly the concentrations of fluorescent proteins respond to increased or decreased heavy metal concentrations.

References

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