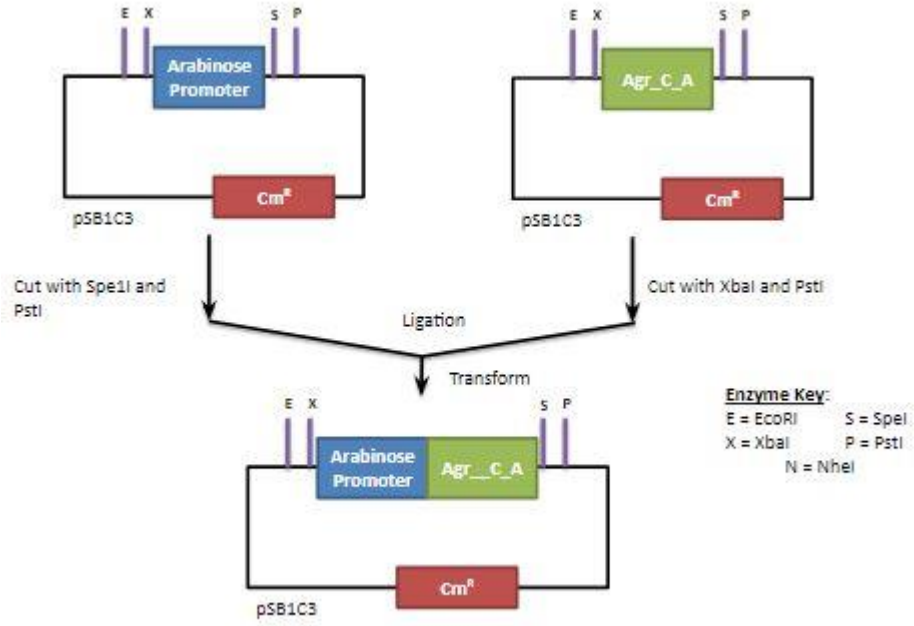
Arabinose Promoter (BBa_I0500) + Agr C & A

Plasmid Generation Protocol



1. Miniprep overnight cultures of **BBa_I0500** (Arabinose Promoter) and **BBa_I746107 (Agr C & A)**. This is your backbone and insert.
2. Digest BBa_I0500 plasmid with **SpeI** and **PstI** at **37ºC** for **60mins**. Use the following mixture:

Molecular Grade Water	Calculated. Use to make reaction total volume = 50 µL
10x NEB Buffer 4	5µL
DNA	1µg
SpeI	0.5µL
PstI	0.5µL

3. Digest BBa_I746107 (Agr C & A) plasmid with **XbaI** and **PstI** at **37ºC** for **60mins**. Use the following mixture:

Molecular Grade Water	Calculated. Use to make reaction total volume = 50 µL
10x NEB Buffer 4	5µL
DNA	1µg
XbaI	0.5µL
PstI	0.5µL

4. Clean up all DNA fragments with PCR clean-up kit.
5. Using the "Ligation Template" excel sheet, calculate the amount of each component to combine in a ligation mix for an Insert:Backbone ratio of **4:1** with total DNA concentration of **7.5-10.0ng/uL**.

Incubate at 25ºC for 1 hr. Heat inactivate at 80 C for 10 mins. Place on ice.

6. **Transformation** (Need 2x LB+Cm20 plates. Check for plates. Check for hockey spreaders/glass beads)**:**
    - Set water bath to 42ºC.

- Remove LB+Cm20 plates from 4°C and allow them to come to RT.
- Thaw chemically competent cells on ice. Leave in microcentrifuge tube.
- Add **5µL** of each ligation mix to individual chemically competent cells.
- Incubate on ice for 30 min.
- Heat shock cells for 60 sec at 42°C without shaking.
- Place on ice for 2 minutes.
- Aseptically (by the fire or in the hood) add **250µL** of SOC media to the tube. Cap tightly.
- Place tube horizontally in shaker. Incubate at 37°C and 225 rpm for 1 to 2 hr.
- In the laminar hood, spread 100uL of transformants onto LB+ Cm20 plates.
- Leave plates in 37°C incubator overnight. Store remaining liquid cultures in 4°C.

7. Next day, pick and annotate (give each colony a #) **16** colonies onto <u>new</u> LB+ Cm20 plates. Place new plates in 37°C overnight.
8. **<u>Verify all 16 colonies</u>** via PCR with **Taq DNA polymerase** using these primers: VF2 and VR. Use following mixture:

Component	50µl reaction
Molecular grade H2O	Added first. 38.5µL
10x Standard Taq Buffer	5µl
10 mM dNTPs	1µl
Sequencing VF2 (10µM)	2.5µl
Sequencing VR (10µM)	2.5µl
Taq DNA polymerase	Added last. 0.5µl

Sequencing VF2: TGCCACCTGACGTCTAAGAA [Tm = 55.9]
Sequencing VR: ATTACCGCCTTTGAGTGAGC [Tm = 55.3]

Use the following thermalcycler protocol:

Cycle Step	3-step protocol		Cycles
	Temp (°C)	Time	
Initial denaturation	95	5 min	1
Denaturation	95	30s	**35**
Annealing	*68*	30s	
Extension	68	*4min 30sec*	
Final Extension	68	10min	1
Hold	4	hold	

9. Run **10µL** of all 16 PCR products onto gel (110V for 60mins). [*Do you see the expected size(s)*? expected size is 1109 bp
10. **If** you find colonies with the aforementioned sizes, **PICK BEST TWO MUTANTS** and start overnight cultures in 2mL LB + Kan50. Leave them to grow in the 37°C incubator overnight.
11. Next morning; miniprep them. Measure their concentrations with the nanodrop. Send them for sequencing.