3A Assembly Lab Menu

Streaking from agar stabs

estimated time: 15 min. active, 16-24 hrs. incubation

Materials needed

- 70% ethanol
- Paper towels
- Lab marker/Sharpie
- Agar Stab: Part A - BBa_J04500 (kit)
- Agar Stab: Part B - BBa_J04650 (kit)
- Inoculating loops (kit)
- LB agar plates – Amp/Kan (kit)

Protocol

1. Clean the lab bench by wiping down with 70% ethanol and paper towels.
2. Part A (BBa_J04500) and Part B (BBa_J04650) are both maintained on pSB1AK3 plasmid backbones, which means they are ampicillin- and kanamycin-resistant. Label the agar plates with the names of Part A and Part B.
3. Use an inoculating loop to transfer some cells from the Part A agar stab to the appropriately labeled Amp/Kan agar plate. There is a hole in each agar stab from where it was inoculated. Dip an inoculating loop into the stab at the same location, and streak the bacteria onto the agar plate in a zig-zag pattern. Using a fresh inoculating loop, streak onto the agar plate again creating a new zig-zag pattern that overlaps the first. This will help ensure that you will have single colonies to pick from. Streak gently, and try not to puncture the agar.
4. Repeat step 3 for Part B. This prevents other bacteria from settling, and growing, on your agar plate.
5. Place the agar plates into the incubator with the agar side facing up, lid facing down (see insert). Incubate the agar plates at 37°C for 14-16 hours. Alternately, incubate at room temperature for 24-30 hours. Once your agar plates have grown up you can store them in your fridge (4°C) until you're ready to grow up your cell culture. Plates can be stored at 4°C for up to 3 weeks.

Growing up cell cultures

estimated time: 30 min. active, 16 hrs. incubation

Materials needed

- 70% ethanol
- paper towels
- Lab marker/Sharpie
- 14ml culture tubes (kit)
- 10ml of LB broth - Amp/Kan (kit)
- Inoculating loops (kit)
- Agar plate: Part A – BBa_J04500
- Agar plate: Part B – BBa_J04650
- Rotator/Shaker

Protocol

1. Clean the lab bench by wiping down with 70% ethanol and paper towels.
2. Remove the agar plates for Part A and Part B from the incubator or 4°C fridge.
3. Label one 14ml culture tube for each Part. Add 5ml of LB broth (with ampicillin and kanamycin) to each culture tube.
4. Use an inoculating loop to pick a single colony from each agar plate and inoculate the LB broth, in the appropriately labeled culture tube. Do not use the same inoculating loop more than once! Press lightly on the snap caps of the 14ml tubes, the caps should be a bit loose to allow for air flow.
5. Incubate for 16 hours at 37°C, in a rotator or shaker. Rotation helps the cells grow faster, and prevents them from settling at the bottom.
6. After incubation, the cell culture should be cloudy. You can now firmly press down on the snap caps to seal the tubes and store the cell culture at 4°C until you're ready to move onto the next step.

Miniprepping

estimated time: 1 hr. active

Materials needed

- QIAprep® Spin Miniprep Kit

Protocol

1. Pellet 1-5 ml bacterial overnight culture by centrifugation at >8000 rpm (6800 x g) for 3 min at room temperature (15-25°C).
2. Resuspend pelleted bacterial cells in 250µl Buffer P1 and transfer to a microcentrifuge tube.(1.5ml)
3. Add 250 ul Buffer P2 and mix thoroughly by inverting the tube 4-6 times until the solution becomes clear. Do not allow the lysis reaction to proceed for more than 5 min. If using LyseBlue reagent, the solution will turn blue.
4. Add 350 ul Buffer N3 and mix immediately and thoroughly by inverting the tube 4-6 times. If using LyseBlue reagent, the solution will turn colorless.
5. Centrifuge for 10 min at 13,000 rpm (~17,900 x g) in a table-top microcentrifuge.
6. Apply the supernatant from step 5 to the QlAprep spin column by decanting or pipetting. • Centrifuge for 30-60 s and discard the flow-through
7. Recommended: Wash the QlAprep spin column by adding 500 ul Buffer PB. • Centrifuge for 30-60 s and discard the flow-through
8. Wash the QlAprep spin column by adding 750 pl Buffer PE. • Centrifuge for 30-60 s and discard the flow-through,
9. Centrifuge for 1 min.
10. Place the QlAprep column in a clean 1.5 ml microcentrifuge tube. To elute DNA, add 50 p1 Buffer EB (10 mM Tris•a, pH 8.5) or water to the center of the QlAprep spin column, let it stand for 1 min, and centrifuge for 1 min.

Restriction digest

estimated time: 30 min. active, 50 min. incubation

Materials needed

- 70% ethanol
- Paper towels
- Ice
- Container for ice
- Lab marker/Sharpie
- pSB1C3 linearized plasmid backbone (25ng/ul) (kit)
- Part A (25ng/ul) (kit)
- Part B (25ng/ul) (kit)
- RFP Control (20ng/ul) (kit)
- 0.6ml tubes (kit)
- NEB buffer 2
- BSA
- NEB enzymes: EcoRI, SpeI, XbaI, PstI
- Thermocycler, or waterbath and thermometer

Protocol

1. Note: to prevent cross contamination make sure to not reuse pipette tips.
2. Clean the lab bench by wiping down with 70% ethanol and paper towels.
3. Keep all enzymes and buffers used in this section on ice.
4. Thaw NEB Buffer 2 and BSA in room temperature water. Re-homogenize both by inverting the tubes, and flick/spin them to collect the liquid at the bottom of the tube.
5. Label four 0.6 tubes: Part A, Part B, pSB1C3 (linearized plasmid backbone), and RFP Control
6. Add 500ng of DNA to the appropriate tube. Add distilled water to the tubes for a total volume of 42.5ul in each tube.
   Calculation example (with 25ng/ul as DNA sample concentration):
   500ng ÷ 25ng/ul = 20ul of DNA sample
   42.5ul (total volume) – 20ul (DNA sample) = 22.5ul of distilled water
7. Pipet 5ul of Buffer 2 to each tube.
8. Pipet 0.5ul of BSA to each tube.
9. In the Part A tube: Add 1ul of EcoRI enzyme, and 1ul of SpeI enzyme.
10. In the Part B tube: Add 1ul of XbaI enzyme, and 1ul of PstI enzyme.
11. In the pSB1C3 tube: Add 1ul of EcoRI enzyme, and 1ul of PstI enzyme.
12. In the RFP Control tube: Add 1ul of EcoRI enzyme, and 1ul of PstI enzyme.
13. The total volume in each tube should be approximately 50ul. Mix well by pipetting slowly up and down 5x. Be gentle, and do not vortex. Spin the samples for 5 seconds in a microcentrifuge, or flick them to collect all of the mixture to the bottom of the tube.
14. Incubate the restriction digests at 37°C for 30 minutes, then 80°C for 20 minutes. We use a thermocycler, but a waterbath and an accurate thermometer works well also!
15. The digested DNA can be stored at 4°C for a few days. For longer storage, keep at -20°C.

	Part A	Part B	pSB1C3 backbone	RFP Control
DNA	20ul	20ul	20ul	25ul
distilled water	22.5ul	22.5ul	22.5ul	17.5ul
NEB Buffer 2	5ul	5ul	5ul	5ul
BSA	0.5ul	0.5ul	0.5ul	0.5ul
Enzyme 1	1ul EcoRI	1ul XbaI	1ul EcoRI	1ul EcoRI
Enzyme 2	1ul SpeI	1ul PstI	1ul Pst1	1ul Pst1

Note: The table assumes you are using the purified DNA samples provided in the 3A Assembly Kit. Part A, Part B, and pSB1C3 at 25ng/ul, and RFP Control at 20ng/ul

Ligation

estimated time: 15 min. active, 50 min. incubation

Materials needed

- 70% ethanol
- Paper towels
- Lab marker/Sharpie
- 0.6 tubes (kit)
- Distilled water (kit)
- Ice
- Container for ice
- T4 DNA Ligase Reaction Buffer
- T4 DNA Ligase
- Microcentrifuge
- Thermocycler, or waterbath and thermometer
- Restriction Digest: pSB1C3 linearized plasmid backbone (see previous step)
- Restriction Digest: Part A (see previous step)
- Restriction Digest: Part B (see previous step)
- Restriction Digest: RFP Control (see previous step)

Protocol

1. Clean the lab bench by wiping down with 70% ethanol and paper towels.
2. Thaw T4 DNA Ligase Reaction Buffer at room temperature. Keep the T4 DNA Ligase in the freezer until you're ready to use it.
3. Label one 0.6ml tube as New Part.
    a. Add 2ul from the pSB1C3 linearized plasmid backbone digest.
    b. Add 3.3ul from the Part A digest.
    c. Add 3.9ul from the Part B digest.
    d. Add 1ul of T4 DNA Ligase Reaction Buffer.
    e. Add 0.5ul of T4 DNA Ligase (keep this at -20°C until use!).
    f. Mix by gently pipetting up and down 3x. Do not vortex; this inactivates the enzymes. Place tube in microcentrifuge for a quick 5 second spin or flick the tube to collect the mixture at the bottom.
4. Label one 0.6 tube as Ligation Control.
    a. Add 2ul from the RFP Control digest.
    b. Add 6.5ul of distilled water.
    c. Add 1ul of T4 DNA Ligase Reaction Buffer.
    d. Add 0.5ul of T4 DNA Ligase.
    e. Mix by gently pipetting up and down 3x. Do not vortex; this inactivates the enzymes. Place tube in microcentrifuge for a quick 5 second spin or flick the tube to collect the mixture at the bottom.
5. Incubate at 16°C for 30 minutes, then at 80°C for 20 minutes. We use a thermocycler, but a waterbath and thermometer combination works great too! The ligated products can be stored at -20°C.

	Ligation: New Part	**Ligation Control**
Digest 1	2ul pSB1C3	2ul RFP Control
Digest 2	3.3ul Part A	-
Digest 3	3.9ul Part B	-
distilled water	0ul	6.5ul
T4 DNA Ligase	0.5ul	0.5ul
T4 DNA Ligase Buffer	1ul	1ul

Transformation

estimated time: 1 hr. active, 12-24 hrs. incubation

Materials

- 70% ethanol
- Paper towels
- Lab marker/Sharpie
- Ice
- Container for ice
- Timer
- NEB10 cells (see Growing step for preparation instructions)
- 2.0ml microcentrifuge tubes (kit)
- Inoculating loops (kit)
- Ligation: New Part (see previous step)
- Ligation: Control (see previous step)
- DNA: RFP Control (20ng/ul) (kit)
- SOC media (kit)
- LB agar plates - Chloramphenicol (kit)
- Sterile glass beads (kit)
- Waterbath and thermometer

Protocol

Note: SOC media gets contaminated easily, so be careful when handling. if possible, wear gloves, and only open the container when you need to.

1. Clean the lab bench by wiping down with 70% ethanol and paper towels.
2. Keep all materials on ice unless otherwise specified! This will help make the cells more competent and easier to transform.
3. Label a 2.0ml microcentrifuge tube as Transformation Control, another as Ligation: New Part, and one more as Ligation Control.
4. Add 5ul of RFP Control DNA (20ng/ul) into the Transformation Control tube.
5. Add 2ul of the New Part ligation product into the Ligation: New Part tube.
6. Add 2ul of the RFP Control ligation product into the Ligation Control tube.
7. Place the tubes on ice to pre-chill them.
8. Thaw one competent cell aliquot tube on ice (this takes about 5-8 minutes).
9. Gently flick the tube of competent cells, then pipet 50ul of competent cells into each 2.0ml microcentrifuge tube.
10. Try to keep the cells as cold as possible by holding just the top of the tube, not the bottom where the cells are.
11. Incubate the DNA and cell mixtures on ice for 30 minutes. During this incubation, pre-heat the waterbath to 42°C.
12. Place the tubes into the waterbath for 60 seconds. Immediately place the tubes back on ice for 5 minutes.
13. Add 200ul of SOC media to each tube. Gently tap the tubes with your finger to mix.

14. Incubate the tubes at 37°C for 2 hours. During this time, prepare the agar plates by labeling them. Add 3 - 6 glass beads per plate.
15. Pipet 200ul of the Transformation Control onto the appropriate plate. Spread evenly over the surface of the agar by gently shaking the plate back and forth. The beads will do the work for you!
16. Repeat step 11 for the other two transformations.
17. Place the agar plates into the incubator with the agar side facing up, lid facing down. Incubate the agar plates at 37°C for 12 - 14 hours. Alternately, incubate at room temperature for 24 hours.
18. Check for red colonies the next day, and post your results online in the next section! Plates can be stored at 4° Celsius for up to two weeks.