Pathfinder Toolkit: Plasmids and Protocols

**Plasmids (Summary of Table 1, Elston et al. 2023)**

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| Plasmid | Origin of Replication | Reporter Gene | Antibiotic Resistance |
| pSL1 | RSF1010 | RCP (mRFP1) | Kan |
| pSL1-GFP | RSF1010 | GFP | Kan |
| pSL2 | RSF1010 | RCP (mRFP1) | Spec |
| pSL3 | RSF1010 | RCP (mRFP1) | Gent |
| pSL4 | RSF1010 | RCP (mRFP1) | Cam |
| pSL5 | RSF1010 | RCP (mRFP1) | Ery |
| pSL6 | RSF1010 | RCP (mRFP1) | Tet |
| pSL7 | RSF1010 | RCP (mRFP1) | Carb |
| pSL9 | pBBR1 | E2C | Kan |
| pSL25 | RP4 | BCP (amilCP) | Kan  |

**Conjugation Protocol**

Day 0

1. Grow overnight cultures of the donor and acceptor strains.
	1. Donor strains must be grown in the presence of DAP (0.3 mM) and antibiotic (if applicable)

Day 1

1. Gently spin down 1 mL of culture (~6800 x g for 1 minute) and decant the supernatant.
	1. If working with plates, scrape growth and resuspend in 1 mL of PBS before spinning down.
2. Resuspend and wash the donor and recipient cells in 1 mL of PBS.
3. Repeat step 1, spinning down the samples once more, decanting the supernatant, and resuspending in 1 mL of PBS.
	1. This removes residual antibiotics from the donor cell culture.
4. Measure optical density (OD600)
	1. Blank with PBS (500 μL)
	2. Combine 250 μL PBS with 250 μL of the sample in a cuvette.
	3. Take OD600 readings.
	4. Multiply the values by 2 for the true OD600 of the sample.
5. Combine a 1:1 ratio of donor and recipient cells in a micro centrifuge tube (100 μL total). Mix by pipetting twice.
	1. Use the Barrick Lab Conjugation Calculator located on the bottom of the conjugation protocol page on the website to determine these values (<https://barricklab.org/twiki/bin/view/Lab/ProtocolsConjugation>)
	2. For slow growing strains that will be outcompeted rapidly by E. coli, a larger acceptor:donor ratio will yield more transconjugants.
		1. I suggest a 1:9 ratio (10 μL of donor E. coli for every 90 μL acceptor, after standardizing to the lowest OD)
6. Divide an LB + DAP plate into quadrants and label it by sample name.
	1. If acceptor bacteria cannot grow on LB, use a plate they can grow on. Be sure that it is supplemented with DAP.
7. Plate 100 μL of each sample onto the non-selective plate containing DAP into each quadrant. Do not spread.
	1. If doing 5+ conjugations, only plate 50 μL.
8. Allow the spots to fully dry before placing them in the incubator for overnight growth.
	1. Plates can be dried quickly by leaving the lid off in a sterile environment (i.e. having the flame on and plates as close as possible).

Day 2

1. Label and fill a microcentrifuge tube with 1 mL of PBS for each sample.
2. Scrape up each conjugation mixture and resuspend it in the PBS by vortex.
3. Gently spin down (~6800 x g for 1 minutes), decant the supernatant, and resuspend in 1 mL of PBS.
4. Dilute the sample(s) to desired level.
	1. For conjugation efficiency / colony counts, serially dilute to 10-7.
	2. If not, 10-3 is sufficient.
5. To serially dilute in a 96 well plate:
	1. Pipette 200 μL of the stock into the first row of a sterile 96 well plate.
	2. Pipette 180 μL of PBS into the second row, continuing until you reach the desired dilution.
	3. Move 20 μL from the stock (row A) into the row below (row B). Mix up and down, then discard the tips. Repeat this process from the second row to the third, third to fourth, and so on.
6. Spot plate 3 μL of dilutions onto selective (antibiotic containing) and non-selective plates.
	1. Non-selective plates are only necessary if conjugation efficiency is being calculated. Otherwise, one selective plate will suffice.
	2. Plating technical replicates (~3) is suggested, especially for conjugation efficiency.
	3. If only some dilutions are being plated (i.e. only up to 10-3), it is possible to plate up to 50 μL of each dilution on the plate if properly spaced out.

Day 3

1. Observe plates and, if applicable, count colonies to calculate conjugation efficiency
	1. Use the spreadsheet linked on the Barrick Lab website (<https://barricklab.org/twiki/bin/view/Lab/ProtocolsCFUCounts>) to determine CFU/μL values.
2. Conjugation Efficiency % = [conjugated]/[total], or [selective CFU]/[non-selective CFU], multiplied by 100.
3. Pick a single colony(s) from the selective plate and streak onto a new selective plate or grow in selective liquid media to make a freezer stock of the conjugated sample the next day.

**Notes**

* Previous work in pseudomonas showed that RSF1010 backbone plasmids were conjugated at a high efficiency, with pBBR1 and RP4 at much lower efficiencies.
* Previous work in *Snondgrassella alvi* showed that the RSF1010 backbone had the highest conjugation efficiency, with pBBR1 slightly lower, and RP4 unable to be conjugated.

**References**

Elston, K.M., Phillips, L.E., Leonard, S.P. *et al.* The Pathfinder plasmid toolkit for genetically engineering newly isolated bacteria enables the study of *Drosophila*-colonizing *Orbaceae*. *ISME COMMUN.* **3**, 49 (2023). <https://doi.org/10.1038/s43705-023-00255-3>