**Supplementary Note:** Detailed protocol for inserting payloads into genome landing pads

**PREPARATION OF LANDING PAD CONSTRUCTS**

Construct plasmids containing the payloads to be inserted in LPattB5, LPattB2, and LPattB7

1. Clone payloads to one of the landing pad integration plasmids using Type IIS assembly with the appropriate scars (w/o OriT: pAJT194-TetR, pAJT196-KanR, pAJT195-CmR; w/ OriT: plYJP068-TetR, plYJP066-KanR, plYJP070-CmR) to target LPattB5, LPattB2, or LPattB7, respectively.
2. Transform the clone product into *E. coli* EC100D pir+ competent cells and sequence verify the construct.
3. MiniPrep plasmids to obtain high concentrations of DNA.
   1. This will help minimize the volumes being added to electrocompetent cells to improve transformation efficiencies.
   2. Multiple MiniPreps can be concentrated into one elution by taking the elution from one MiniPrep and using it to elute a separate column containing the same plasmid.
   3. Do MaxiPrep
      1. For frequently used plasmids, such as the integrase plasmid (plYJP053), we had good success with MaxiPrep services from Quintara Biosciences, yielding >1 mg DNA

**PREPARATION OF ELECTROCOMPETENT CELLS CONTAINING LANDING PADS**

1. Streak cells harboring empty landing pads (EcN AJT202) on a 2% LB-agar plate without antibiotics and incubate overnight at 37oC.
2. Pick a single colony and inoculate into 2 mL LB media with antibiotics and incubate overnight at 37oC, shaking at 250 rpm.
3. Dilute cells 50-fold 1 mL into 50 mL LB no salt medium (1 L water + 10 g/L tryptone + 5 g/L yeast extract, autoclaved) in a 250-mL Erlenmeyer flask.
   1. Note: In LB no salt media, we have observed that some strains are sensitive to antibiotics despite containing antibiotic resistance genes. Since all of our constructs are integrated onto the genome, where antibiotic selection is not required for maintaining synthetic constructs, we have chosen to omit antibiotics when using LB no salt media, even if antibiotic resistance markers are present.
   2. Note: 50 mL of culture will yield 5-10 transformations. For larger batches of competent cells, adjust volumes for overnights and subcultures accordingly. For more difficult-to-transform constructs, cells can be more concentrated to yield fewer, yet higher-efficiency transformations.
4. Grow for one-two hours until OD600 = 0.55-0.65 is reached.
5. While cells are growing:
   1. Prepare ice/water bath.
      1. Pre-chill the appropriate number of 50-mL Falcon tubes and electroporation cuvettes in ice/water bath.
      2. Pre-chill 10% glycerol for washes.
      3. If making frozen aliquots,
         1. Note: Frozen competent cells lead to decreased transformation efficiencies compared to freshly prepared cells. Attempt transformations with freshly prepared cells to get an idea of competency before attempting with frozen cells.
         2. Pre-chill 1.5-mL microcentrifuge tube or 200-µL PCR strip tubes
         3. Check liquid nitrogen.
   2. Pre-chill centrifuge to 4°C.
   3. Pour plates if doing transformations same day.
6. Transfer cells to pre-chilled 50-mL Falcon tubes.
7. Centrifuge cells at 4000g and 4°C for 10 minutes.
8. Remove the supernatant.
9. Resuspend cell pellet in 4 mL of ice-chilled 10% glycerol.
10. Centrifuge cells at 4000g and 4°C for 10 minutes.
11. Remove the supernatant.
12. Repeat Steps 9-11 twice more for a total of three glycerol washes.
13. Resuspend cell pellet in 500 μL of ice-chilled 10% glycerol, yielding electrocompetent cells.
    1. This yields five 100 μL aliquots of competent cells. Volume can be adjusted to yield more or less concentrated aliquots to provide fewer or more transformations. Number of colonies will correlate with concentration of cells in competent cell aliquots.
14. If making frozen aliquots, which we have found decreases transformation efficiency, aliquot 100 µL cells into pre-chilled 200-µL PCR strip tubes. Each 100 µL aliquot will yield a single transformation.

**TRANSFORMATION WITH PAYLOAD DNA**

1. Aliquot 100 µL cells into pre-chilled electroporation cuvettes.
2. Dialyze 1500 ng of the integrase plasmid (plYJP053 or plYJP022-int7) and 500-2000 ng of the DNA containing the plasmid with payload.
   1. Dialyzing DNA helps to remove salts that impede electroporation.
   2. If >10 μL of DNA is required to achieve the desired amount of DNA to be added to a transformation, pipette DNA into a removable dialysis device from a Pierce 96-well microdialysis plate with 10K molecular weight cutoff (Thermo Scientific, USA, 88260).
   3. Next, pipette 80 μL of air into the device to push the DNA further into the device for proper dialysis.
   4. Place each device in 1.8 mL of water in a 2 mL deep-well 96-well plate (USA Scientific, USA, 1896-2000) and allowed to dialyze for 30 minutes while preparing electrocompetent cells.
   5. Remove DNA by removing the device from water, shaking with the flick of the wrist to collect the DNA at the bottom of the device, and subsequently pipetting out 100 μL of air and DNA.
3. Add the dialyzed plasmids to the prepared competent cells
4. Electroporate the mixture (Using the Eppendorf Electroporator 2510, we found that 2500 mA and a time constant after the transformation 4.0-6.0 worked best).
5. Immediately after electroporation, add 400 µL of SOC recovery media to the cells.
6. Incubate the cells at 30oC for 1-2 hours and then spread on a 2% LB agar plate with appropriate antibiotic (Insertion in landing pad LPattB5, LPattB2, or LPattB7 was selected with 5 μg/mL Tet, 50 μg/mL Kan, or 34 μg/mL Chlor, respectively).
7. Incubate overnight at 37oC.
8. Pick colony and inoculate in 200 µL LB media with appropriate antibiotic for 3 hours. Typically, we pick three colonies for a higher likelihood of identifying a successful integration.

**PCR SCREENING FOR SUCCESSFUL INTEGRATION**

1. Primers that can amplify the entire genomic insertion were used to validate the integration of the payload. The following primers are used at concentrations of 10 µM. If integrations were >5 kb, alternative primers that can amplify the junction between integrated constructs and the adjacent genomic DNA were used.
   1. LPattB5: ogAJT0287 (GTGACAGAGAAAAAGTAGCCGAAGATG) ogAJT0288 (GCGTAACCTGGCAAAATCGGT)
   2. LPattB2: ogAJT0283 (CCCGAATAAACGGTCTCAGCC) ogAJT0284 (GCTTTGTGCTGGAAGATAAGCTGATTC)
   3. LPattB7: ogAJT0300 (GGCTTGCCATATCGTTCATTCAGTTTC) ogAJT0301 (GAGCGCACATTAACGGCC)
2. Perform the PCR reaction. Our preference is NEB Q5 High-Fidelity 2X Master Mix (M0492S) with the following volumes: 12.5 μL Q5 2X Master Mix, 9 μL water, 1.25 μL Primer #1, 1.25 μL Primer #2, 1 μL of colony picked into water or overnight culture. The thermal cycler parameters are: 5 min initial denaturation (98°C), 5s denaturation (98°C), 30s anneal (60°C), 30s-1 min/kb extension (72°C), repeat denaturation/anneal/extension steps 34 times, 30s-1 min/kb final extension (72°C).
3. Confirm the amplicon size using gel electrophoresis by adding 5 µL of PCR product into 1% agarose gel. NEB 1kb ladder was run together to confirm the size.

**REMOVAL OF ANTIBIOTIC RESISTANCE MARKERS**

1. Inoculate the 170 µL culture from Step 21 to 4 mL SOB media without antibiotics for 3 hours at 37oC.
2. Once cells reach OD600=0.4, Centrifuge cells at 4700g and 4oC for 10 minutes.
3. Remove the supernatant.
4. Add 1 mL of ice-chilled 10% glycerol to the cell pellet.
5. Transfer the resuspended cells into a 1.5 mL Eppendorf tube and centrifuge at 21000g and 4oC for 30 seconds.
6. Remove the supernatant.
7. Repeat steps 30 to 31.
8. Add 20 ng of the FLP encoding plasmid (pE-FLP)
9. Electroporate the mixture using the Eppendorf Electroporator 2510 at 2500 mA with time constant after the transformation >4.0.
10. Immediately after electroporation, add 1 mL of SOC media to the cells.
11. Incubate the cells at 30oC for 1 hour and then spread on a 2% LB agar plate with 100 µg/mL Carb.
12. Incubate the cells overnight at 30oC
13. Pick colony and streak the colony on 2% LB agar plate with no antibiotics.
14. Pick 5-6 colonies from the streak after 14 hours of incubation and resuspend each to 30 µL LB media without antibiotics.
15. Inoculate the resuspended cells into 5 different media by adding 5 µL of resuspended cells. The five media compositions are: LB media without antibiotics, with Amp 100 µg/mL, Kan 50 µg/mL, Cm 35 µg/mL, Tet 5 μg/mL. Incubate at 37°C overnight.
16. Find colonies that only grew on LB media without antibiotics.
17. Take 30 µL of the cultures from Step 41 and incubate them at 98oC for 10 minutes.
18. Repeat PCR screening steps to confirm the loss of the antibiotic resistance marker.
19. Once the size and antibiotic sensitivity is confirmed, add 25% autoclaved glycerol (v/v) to create glycerol stock.
20. Store glycerol stock at -80°C.