

# **TAC–TIC, a high-throughput genetics method to identify triggers or blockers of bacterial toxin–antitoxin systems**

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# **TAC–TIC, a high-throughput genetics method to identify triggers or blockers of bacterial toxin–antitoxin systems**

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## **Supplementary Methods**

### **Construct conjugation gene-donor libraries from phages (Timing 4-6 weeks)**

**CRITICAL** Follow this procedure only when making a new conjugation donor gene library.

#### **Clone phage genes into pJB158 by Golden Gate assembly (Timing 1-2 weeks)**

1. Design and order forward and reverse PCR primers for every gene of your source phage genome with BsaI-containing overhangs (Supplementary Table 1 for primer design). If the gene to be amplified contains BsaI sites, use alternative cloning methods (e.g., Gibson Assembly or AvrII-FseI restriction enzymes for pJB158).

**CRITICAL STEP** Order the forward and reverse primers for each phage gene pre-arrayed in the same well of two separate 96-well plates (two plates can take 3-6 weeks to arrive).

**PAUSE POINT** The primer plates can be kept at -20°C until required.

2. Extract DNA from your phage of interest<sup>1</sup> and measure its concentration with a NanoDrop or a Qubit fluorometer.

3. Use a high-fidelity polymerase (Q5® High-Fidelity DNA Polymerase) to PCR amplify each gene from your source phage in 96-well PCR plates (Supplementary Fig. 2):

Component	Volume (μL)	Final Amount or Conc.
Phage genomic DNA	1	< 1 ng
Q5 Reaction buffer	5	1X
dNTPs mix	1	0.2 mM
Forward Primer	2.5	10 nM
Reverse Primer	2.5	10 nM
Q5® High-Fidelity DNA Polymerase	0.25	0.02 U/μL
Nuclease-free water	Up to 25	
Total	25	

For 96-well plate PCRs (1-2 plates are usually sufficient to cover an entire phage genome), prepare on ice a master mix with all PCR components except for the primers (unique for each well), and transfer 20 μL in each well using an automatic stepper pipette. Then, transfer 2.5 + 2.5 μL from 100 nM stocks of the forward and reverse primers (Step 1) with a 10 μL multichannel pipette into the final PCR plate, and run the PCR:

PCR step	Temp	Cycle time
Initial Denaturation	98°C	5 min
	98°C	10 sec
35 cycles	60°C	30 sec
	72°C	1 min/kilobase
Final Extension	72°C	10 min
Hold	4°C	Forever

**CRITICAL STEP** Use primers at 10 nM to reduce the formation of primer dimers, which compete for ligation with the desired PCR product, leading to misassembled vectors without PCR inserts.

**PAUSE POINT** PCR products can be stored at 4°C for weeks and at -20°C for longer-term.

4. Run 5 μL from each PCR (with loading dye and water to a final volume of 12 μL per sample) on an agarose gel to verify that the PCRs worked, and that there is a single DNA band of the expected size per phage gene.

5. If PCRs are successful, clone your PCR products into the pJB158 vector by Golden Gate assembly in 96-well PCR plates (Supplementary Fig. 2). Prepare on ice a master mix containing all the components shown below, except for the PCR products (unique for each reaction), mix well, and pipette 10  $\mu$ L in each well using an automatic stepper pipette.

Component	Volume ( $\mu$ L)	Final amount or Conc.
Destination vector (pJB158)	X	50 ng
PCR reactions	0.6	>3:1 excess of vector (molar ratio)
Bsal-HF@v2	0.4	8 units
T4 ligase	0.4	160 units
T4 ligase buffer	1	1X
MQ water	Up to 10	
Total	10	

Then, use a 10  $\mu$ L multichannel pipette to add 0.6  $\mu$ L of the unpurified PCR products from the PCR plates (Step 4) to the Golden Gate assembly plates. Seal the plate with a cover foil and run the Golden Gate Assembly 96-well plate reaction in a thermocycler:

Step description	Temperature/Time	Number of cycles
Bsal cuts	37°C/2 min	30
T4 ligase joins DNAs	16°C/5 min	
Ligase inactivation	60°C/1 min	1
Bsal inactivation	80°C/20 min	1
Hold	4°C	Forever

**CRITICAL STEP** Include a negative control Golden Gate reaction (without PCR product).

**CRITICAL STEP** The volume of the non-purified PCR products must be kept below 1  $\mu$ L per reaction, as they contain DNA polymerase that can fill-in the overhangs produced by Bsal (producing misassembled vectors).

**CRITICAL STEP** The pJB158 vector replicates at ~15 copies/cell when introduced in *E. coli* DATC cells (or other *pir*<sup>+</sup> strains) but replicates at 1 copy/cell in cells without *pir*<sup>+</sup>. To get sufficient plasmid DNA for the Golden Gate Assembly, do a midiprep from an *E. coli* DATC transformed with pJB158 (ZymoPURE™ II Plasmid Midiprep Kit).

**PAUSE POINT** Golden Gate assembly products can be stored at -20°C for long-term.

**Prepare competent *E. coli* DATC cells (Timing 2 d)**

6. Streak-out *E. coli* DATC from a glycerol stock onto an LB-DAP agar plate and incubate the plate at 37°C overnight (12-16 hours).

7. Inoculate a single colony of *E. coli* DATC in 5 mL of LB-DAP and incubate the culture in a rotating drum roller at 37°C overnight (12-16 hours).

8. Inoculate 55 µL from the overnight *E. coli* DATC culture in a sterile 0.5 L flask containing 110 mL of LB-DAP, incubate the culture at 37°C in a shaking incubator (180 rpm), and monitor until it reaches an OD<sub>600</sub> = 0.4-0.5 (~3-4 hours).

**CRITICAL STEP** Perform Steps 9-13 on ice, while using ice-chilled buffers, and pre-cooled centrifuges.

9. Chill the grown culture (Step 8) in an ice bucket for 5 minutes.

10. Transfer two 50 mL aliquots of the chilled DATC culture in two 50 mL Falcon tubes and use a benchtop centrifuge to spin the tubes at 960 g for 10 minutes at 4°C.

11. Discard the supernatant and resuspend the cell pellets first in 1 mL of TBF1 buffer, and then top up with 39 mL of TBF1 buffer. Keep the resuspended cells on ice for 5 minutes.

12. Centrifuge the cells at 960 g for 10 minutes at 4°C.

13. Discard the supernatant and resuspend the cell pellets in 4 mL of TBF2. Keep the resuspended cells on ice for 15 minutes. Use an automatic stepper pipette to aliquot 50 µL of the resuspended *E. coli* DATC cells into each well of a 96-well PCR plate. Seal the plates using a cover foil and freeze the competent cell plates at -80°C.

**PAUSE POINT** Competent cells can be stored at -80°C for up to 6 months.

**Transform the *E. coli* DATC competent cells with the Golden Gate assembly reactions (Timing 2 d)**

**CRITICAL STEP** Before proceeding to Step 14 verify that the *E. coli* DATC cells from Step 13 are competent by transforming them with 50 ng of the destination pJB158 vector (or water as negative control), plating the reactions on selective LB-DAP-tetracycline agar plates, and observing hundreds of tetracycline-resistant colonies growing after an overnight incubation (no colonies should grow in the negative control plates).

14. Thaw the 96-well PCR plates of *E. coli* DATC competent cells (Step 13) and the 96-well Golden Gate assembly reaction plates (Step 5) on ice for 30 minutes.

15. Centrifuge the plates on a benchtop centrifuge at 68 g for 2 minutes at 4°C to spin down the condensation on the cover foils.

16. While working on ice, use a multichannel pipette to transfer 5 µL of the Golden Gate assembly reactions to the *E. coli* DATC competent cells, mix briefly by pipetting up and down, seal the plates with a cover foil, and keep the plate on ice for 30 minutes.

17. Preset a 96-well thermocycler to 42°C and its lid at 50°C. Insert the 96-well plate (Step 16) and heat shock the cells for 45 seconds (keep track of time with a timer).

**CRITICAL STEP** Higher thermocycler lid temperature than 50°C might kill the cells.

18. Quickly transfer the plate on ice and keep the cells on ice for 2 minutes.

19. Fill each well of a 1 mL 96 deep-well plate with 600 µL of LB-DAP medium.

20. Remove the cover foil from the plate (Step 18) and use a multichannel pipette to transfer 55 µL of the transformed competent cells into the 96 deep-well plate (Step 19). Mix well and seal the plate with a sterile gas permeable membrane (AeraSeal film). Incubate the plates in a shaking incubator (600 rpm) at 37°C for 1 hour.

21. Use ~10 glass beads per plate to spread 200 µL from each well of the 96 deep-well plate (Step 20) onto individual LB-DAP tetracycline agar plates. Use a funnel to remove the glass beads from the plates into a waste bottle (as described in Step 26 of main Procedure) and dry the Petri dishes around the updraft of a Bunsen burner (or within a laminar flow cabinet). Incubate the dried plates at 37°C overnight (18-24 hours).

22. The next day, check for colonies on the plates (Supplementary Fig. 2).

## **?TROUBLESHOOTING**

**PAUSE POINT** Store the grown plates in a cold room at 4°C for up to 4 weeks.

**CRITICAL STEP** The number of colonies growing from the negative control Golden Gate reaction (from Step 5) indicates non-digested or re-circularized vectors. If the cloning worked optimally, the number of colonies on experimental plates should be 5-100x times greater than on control plates.

## **Verify phage-gene containing clones by colony PCR (Timing 1-2 weeks)**

23. The empty pJB158 vector has a GFP-gene cloned between the BsaI sites as a selection marker (Supplementary Fig. 2). Mark and avoid GFP-positive colonies by checking the transformation plates under a blue light transilluminator. Select GFP-negative colonies (Step 22) and label them on the back side of the plate using a marker (e.g., for the 4 colonies picked for gene A, label them A1, A2, A3, and A4).

**CRITICAL STEP** This labeling step is important to backtrack the colony-PCR positive colonies.

24. To check that the cloned genes are of the expected size, use primers JB565 and JB566 that anneal on pJB158 (Table 1) to screen four colonies from each individual plate (Step 22) by colony PCR in 96-well PCR plates. Prepare on ice a master mix containing components as shown below (except the template DNA), mix, and pipette 10  $\mu$ L in each well using an automatic stepper pipette.

Component	Volume ( $\mu$ L)	Final amount or Conc.
Colony DNA	/	/
GoTaq® Green Master Mix	5	1X
Forward Primer	0.2	100 nM
Reverse Primer	0.2	100 nM
MQ water	4.6	
Total	10	

25. Use sterile 200  $\mu$ L plastic tips to pick colonies from each plate and drop the tips inside the corresponding PCR plate wells. Once a plate is filled with picked colonies, use a multichannel pipette to discard all the tips, while dispensing any aspirated liquid back in the wells. Seal the 96-well PCR plates with a cover foil and use a 96-well thermocycler to run the PCR reactions.

26. Run 10  $\mu$ L from each colony PCR on an agarose gel to verify that there is a single band of the correct size for each gene. Note down the PCR-positive colonies.

## ?TROUBLESHOOTING

**CRITICAL STEP** You should have at least two colony-PCR positive clones for each gene of your phage library. If more than two colonies are PCR positive, select two clones. If less than two colonies are PCR positive, continue screening until two positive clones are identified.

## Array the phage-gene plasmid donor libraries in 96-well plates (Timing 2 d)

27. Prepare 96-well plate maps indicating the location of each clone of the phage-gene library.

**CRITICAL STEP** Perform Steps 28-33 around the updraft produced by the flame of a Bunsen burner.

28. Fill as many 96 deep-well plates required to array the entire library, with 600  $\mu$ L of LB-DAP-tetracycline. For example, a library size of 170 genes is equal to 340 picked colonies (2 colony-PCR positive clones) and will require four 96 deep-well plates (A, B, C, and D).

29. Return to the selection plates (Step 22) and use sterile 200  $\mu$ L pipette tips to carefully pick once more the colony-PCR positive colonies (verified in Step 26). Drop the tips into each well of the 1 mL 96 deep-wells as designated. Once a plate is filled with picked colonies, use a multichannel pipette to remove all the tips, while releasing the liquid back into the wells.

**CRITICAL STEP** Include in your library at least 6 *E. coli* DATC strains transformed only with the pJB158 vector (per 96-well plate). These are later used as negative controls in TAC–TIC.

30. Seal the 96 deep-well plates with gas permeable membranes (AeraSeal film) and incubate the plates at 37°C in a shaking incubator set at 600 rpm overnight (16-24 hours).

31. The next day, fill 96-well plates with 40  $\mu$ L of 30% sterile glycerol. For each culture plate (Step 30) make two 96-well glycerol replicate plates. For example, if you have four 96 deep-well plates (A, B, C, and D), you need eight glycerol-containing 96-well plates (A1/A2, B1/B2, C1/C2, and D1/D2).

32. Remove the membrane from the overnight culture plates (Step 30) and transfer 40  $\mu$ L from each 96-deep well plate to two 96-well glycerol plates.

33. Seal the 96-well conjugation-donor library plates with cover foils and label them appropriately (name of the plate, construction date, phage source, and responsible lab personnel initials), and store the plates at -80°C (Supplementary Fig. 2).

**PAUSE POINT** The conjugation donor library plates can be kept at -80°C indefinitely.

**CRITICAL STEP** Sequence the gene-donor library to ensure that the cloned sequences are as expected, either by PCR-amplifying inserts with primers JB563/JB564 (Supplementary Table 1), and then Sanger sequencing these amplicons with nested primers JB565/JB566 (Supplementary Table 1), or alternatively, use plate/row/column pooling to sequence pooled insert amplicons on NGS platforms, followed by pool deconvolution<sup>2</sup>.

## References

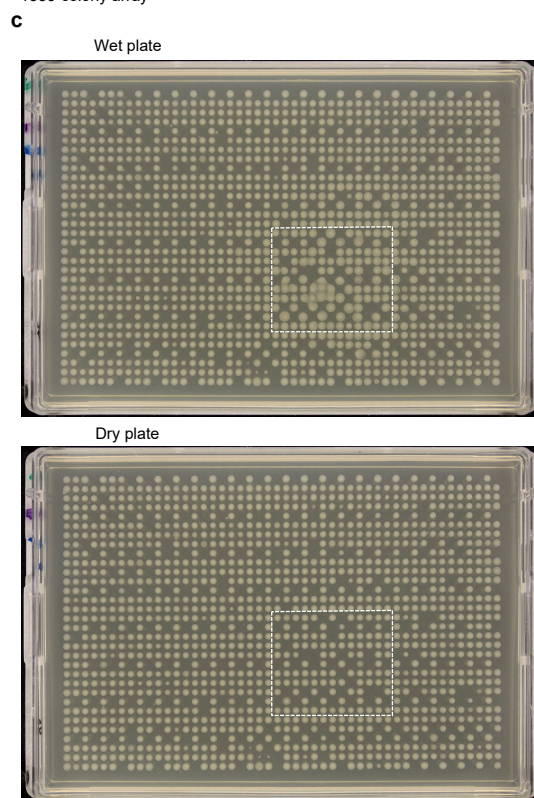
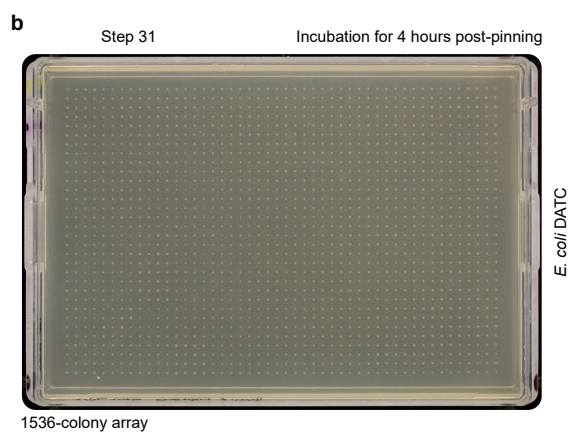
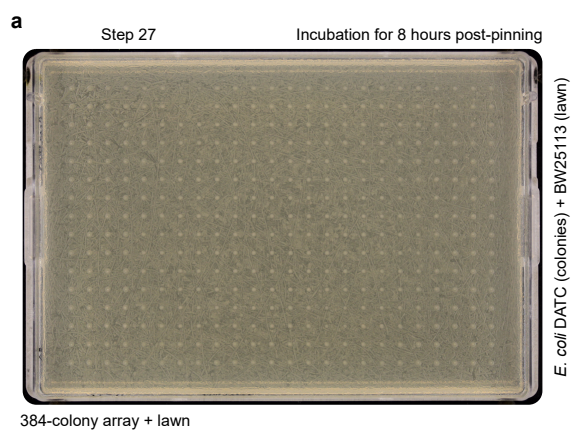
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2. Anzai, I. A., Shaket, L., Adesina, O., Baym, M. & Barstow, B. Rapid curation of gene disruption collections using Knockout Sudoku. *Nat. Protoc.* **12**, 2110–2137 (2017).

## Supplementary figure legends

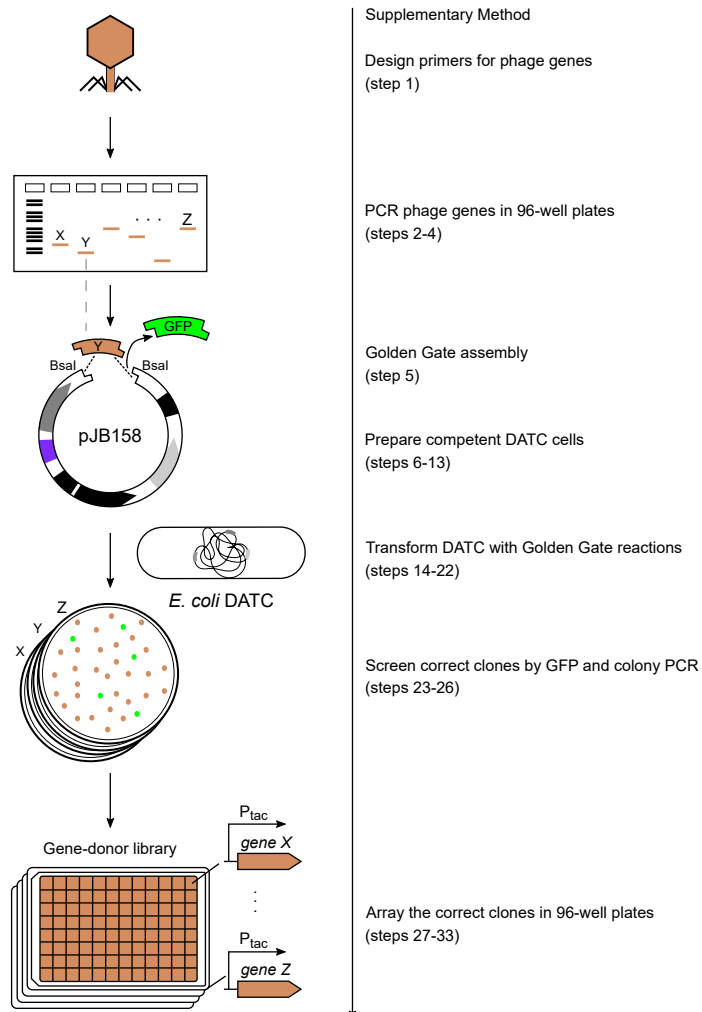
**Supplementary Fig. 1 | Ideal growth state at Steps 27 and 31 of the TAC–TIC procedure.** **a**, To ensure equal conjugation rates within and between plates, lawns of *E. coli* BW25113 conjugation recipients should be confluent across the whole plate, while colony-arrays of *E. coli* DATC gene donors (here in 384-format) should be visibly growing on top of the lawn. **b**, Reducing the inoculum size from the third round of transconjugant selection plates to the final plates, by incubating the colonies for a short duration (4 hours), minimizes overgrowth of outer row/column colonies compared to inner colonies. **c**, Agar plates with wet surfaces lead to colonies fusing (top plate, white box) which confound fitness measurements by Iris, while colony fusion does not occur when using adequately-dried plates (bottom plate, white box).

**Supplementary Fig. 2 | Phage gene-donor conjugation library construction overview.** **Steps 1-4**, Phage DNA is used to PCR amplify each phage-gene (X to Z). **Step 5**, Crude PCR phage-gene amplicons (X to Z) are cloned into the mobilizable pJB158 vector via Golden Gate assembly. **Steps 6-22**, Competent *E. coli* DATC are transformed with the Golden Gate reactions and correct clones are selected by lack of GFP signal and verified via colony-PCR. **Steps 27-33**, Phage gene-donor conjugation libraries are assembled by arraying two colony PCR-verified clones of each phage-gene (X to Z) into 96-well plates.

Supplementary Table 1   Oligos used in Supplementary Method (Steps 1 and 24)		
Name	Sequence (5' → 3')	Description
Forward gene X	GCGAGggtctcgGCATNNNNNG/C	UPPER CASE: Buffer sequence, lower case: BsaI recognition site, <u>Underline</u> : Upstream sticky end for pJB158, <b>Bold</b> : 15-25 bp complementarity to gene start, <i>Italics</i> : G/C clamp
Reverse gene X	GATCTGggtctcgCTTANNNNNG/C	UPPER CASE: Restriction enzyme buffer sequence, lower case: BsaI recognition sequence, <u>Underline</u> : Downstream sticky end for pJB158, <b>Bold</b> : 15-25 bp complementarity to gene end, <i>Italics</i> : G/C clamp
JB563	AATGAGGGCATCGTTCCCAC	Anneals 536 bp upstream of BsaI site in pJB158
JB564	CGCATACGCTACTTGCATTACAG	Anneals 551 bp downstream of BsaI in pJB158
JB565	CAGTGATAACGGACCGCG	Anneals 153 bp upstream of BsaI site in pJB158
JB566	CCGAGCGTTCTGAACAAATC	Anneals 134 bp downstream of BsaI in pJB158



**Supplementary Figure 1**



**Supplementary Figure 2**