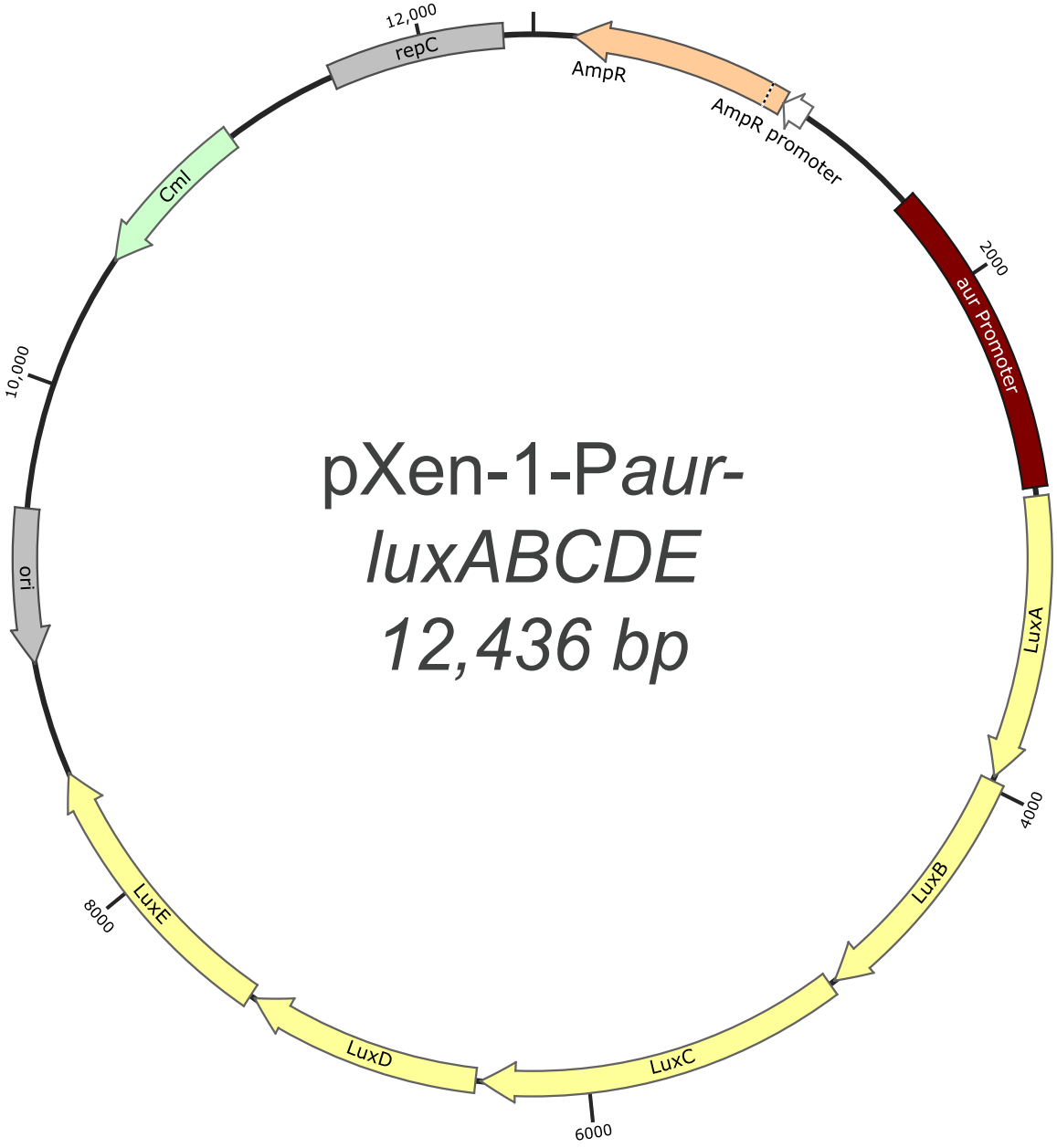
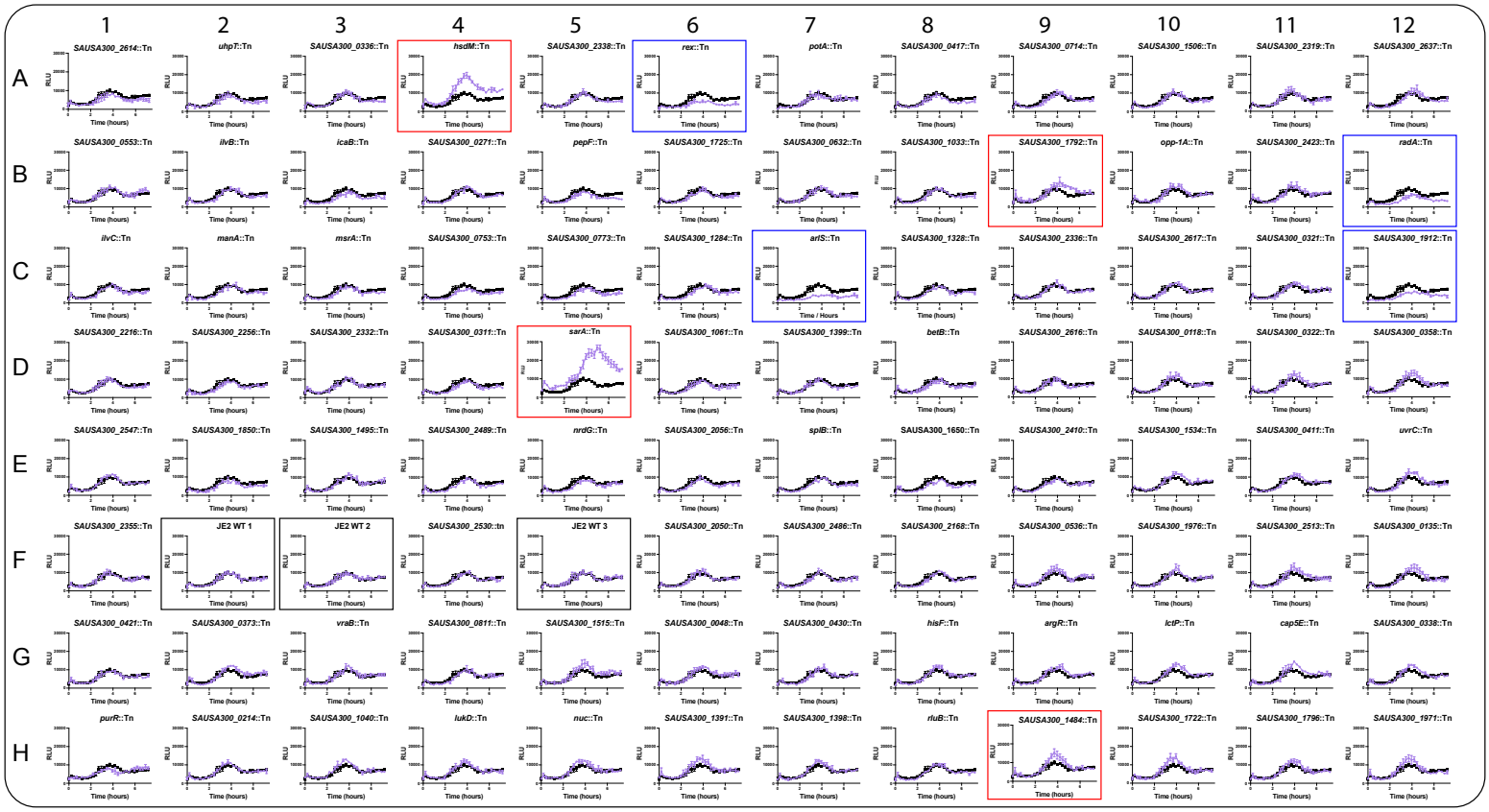



**Supplemental Figure 1: Map of pXen-1 plasmid harboring the *aur* promoter.**




**Supplemental Figure 2: Raw Relative Light Units (RLU) for screen depicted in Figure 2 A.**

**Supplemental Table 1: Strains screened for the proof-of-concept study**

| NTML | Well | Locus Tag | Gene | Product |
|---|---|---|---|---|
| NE1153 | A1 | SAUSA300_2614 | | putative lipoprotein |
| NE1154 | A2 | SAUSA300_0216 | *uhpT* | hexose phosphate transport protein |
| NE1155 | A3 | SAUSA300_0336 | | uncharacterized protein |
| NE1156 | A4 | SAUSA300_0405 | *hsdM* | DNA-methyltransferase |
| NE1157 | A5 | SAUSA300_2338 | *nreB* | nitrogen regulation protein B; Histidine Kinase |
| NE1158 | A6 | SAUSA300_1999 | *rex* | redox-sensing transcriptional repressor |
| NE1159 | A7 | SAUSA300_0999 | *potA* | spermidine/putrescine import ATP-binding protein |
| NE1160 | A8 | SAUSA300_0417 | | uncharacterized tandem lipoprotein |
| NE1161 | A9 | SAUSA300_0714 | | integral membrane protein |
| NE1162 | A10 | SAUSA300_1506 | | uncharacterized protein |
| NE1163 | A11 | SAUSA300_2319 | | ferredoxin-NADP reductase |
| NE1164 | A12 | SAUSA300_2637 | | uncharacterized protein |
| NE1165 | B1 | SAUSA300_0553 | | uncharacterized protein |
| NE1166 | B2 | SAUSA300_2007 | *ilvB* | acetolactate synthase, large subunit |
| NE1167 | B3 | SAUSA300_2601 | *icaB* | biofilm polysaccharide intercellular adhesion deacetylase |
| NE1168 | B4 | SAUSA300_0271 | | ABC transporter, ATP-binding protein |
| NE1169 | B5 | SAUSA300_0902 | *pepF* | oligoendopeptidase F |
| NE1170 | B6 | SAUSA300_1725 | | transaldolase |
| NE1171 | B7 | SAUSA300_0632 | | uncharacterized protein |
| NE1172 | B8 | SAUSA300_1033 | *isdF* | iron-regulated surface determinant protein F |
| NE1173 | B9 | SAUSA300_1792 | | uncharacterized protein |
| NE1174 | B10 | SAUSA300_2411 | *opp-1A* | oligopeptide permease, peptide binding protein |
| NE1175 | B11 | SAUSA300_2423 | | uncharacterized protein |
| NE1176 | B12 | SAUSA300_0511 | *radA* | DNA repair protein |
| NE1177 | C1 | SAUSA300_2009 | *ilvC* | ketol-acid reductoisomerase (NADP+) |
| NE1178 | C2 | SAUSA300_2577 | *manA* | mannose-6-phosphate isomerase |
| NE1179 | C3 | SAUSA300_2594 | *msrA* | peptide methionine sulfoxide reductase |
| NE1180 | C4 | SAUSA300_0753 | | Epimerase family protein |
| NE1181 | C5 | SAUSA300_0773 | | putative staphylocoagulase |
| NE1182 | C6 | SAUSA300_1284 | *cvfB* | conserved virulence factor B |
| NE1183 | C7 | SAUSA300_1307 | *arlS* | signal transduction histidine kinase of TCS ArlRS |
| NE1184 | C8 | SAUSA300_1328 | *norB* | quinolone resistance protein |
| NE1185 | C9 | SAUSA300_2336 | | transcriptional regulator, MerR family |
| NE1186 | C10 | SAUSA300_2617 | | putative cobalt ABC transporter, ATP-binding protein |
| NE1187 | C11 | SAUSA300_0321 | | uncharacterized protein |
| NE1188 | C12 | SAUSA300_1912 | | putative membrane protein |
| NE1189 | D1 | SAUSA300_2216 | | transcriptional regulator, MarR family |
| NE1190 | D2 | SAUSA300_2256 | | putative N-acetylmuramoyl-L-alanine amidase |
| NE1191 | D3 | SAUSA300_2332 | | heat shock protein |
| NE1192 | D4 | SAUSA300_0311 | | PfkB family carbohydrate kinase |
| NE1193 | D5 | SAUSA300_0605 | *sarA* | staphylococcal accessory regulator A |
| NE1194 | D6 | SAUSA300_1061 | | putative exotoxin 3 |
| NE1195 | D7 | SAUSA300_1399 | | phiSLT ORF110-like protein |

| | | | | |
|---|---|---|---|---|
| NE1196 | D8 | SAUSA300_2546 | *betB* | Glycine betaine aldehyde dehydrogenase |
| NE1197 | D9 | SAUSA300_2616 | | cobalt transport family protein |
| NE1198 | D10 | SAUSA300_0118 | *sbnA* | N-(2-amino-2-carboxyethyl)-L-glutamate synthase |
| NE1199 | D11 | SAUSA300_0322 | | NADH-dependent flavin oxidoreductase, Oye family |
| NE1200 | D12 | SAUSA300_0358 | | 5,10-methylenetetrahydrofolate reductase |
| NE1201 | E1 | SAUSA300_2547 | | HTH-type transcriptional regulator |
| NE1202 | E2 | SAUSA300_1850 | | uncharacterized protein |
| NE1203 | E3 | SAUSA300_1495 | | uncharacterized protein |
| NE1204 | E4 | SAUSA300_2489 | | antibiotic transport-associated protein-like protein |
| NE1205 | E5 | SAUSA300_2550 | *nrdG* | anaerobic ribonucleoside-triphosphate reductase-activating protein |
| NE1206 | E6 | SAUSA300_2056 | | uncharacterized protein |
| NE1207 | E7 | SAUSA300_1757 | *splB* | serine protease B |
| NE1208 | E8 | SAUSA300_1650 | | uncharacterized protein |
| NE1209 | E9 | SAUSA300_2410 | | oligopeptide ABC transporter, permease protein |
| NE1210 | E10 | SAUSA300_1534 | | uncharacterized protein |
| NE1211 | E11 | SAUSA300_0411 | | uncharacterized lipoprotein |
| NE1212 | E12 | SAUSA300_1045 | *uvrC* | excinuclease ABC subunit C |
| NE1213 | F1 | SAUSA300_2355 | | putative lipoprotein |
| | F2 | JE2 wild type | | |
| | F3 | JE2 wild type | | |
| NE1216 | F4 | SAUSA300_2530 | | transcriptional regulator, TetR family |
| | F5 | JE2 wild type | | |
| NE1218 | F6 | SAUSA300_2050 | *tenA* | aminopyrimidine aminohydrolase |
| NE1219 | F7 | SAUSA300_2486 | *clpL* | ATP-dependent Clp protease ATP-binding subunit |
| NE1220 | F8 | SAUSA300_2168 | | uncharacterized protein |
| NE1221 | F9 | SAUSA300_0536 | *hchA* | protein/nucleic acid deglycase |
| NE1222 | F10 | SAUSA300_1976 | | succinyl-diaminopimelate desuccinylase |
| NE1223 | F11 | SAUSA300_2513 | | uncharacterized protein |
| NE1224 | F12 | SAUSA300_0135 | *sodM* | superoxide dismutase (Mn/Fe) |
| NE1225 | G1 | SAUSA300_0421 | | uncharacterized protein |
| NE1226 | G2 | SAUSA300_0373 | | uncharacterized protein |
| NE1227 | G3 | SAUSA300_0560 | *vraB* | acetyl-CoA c-acetyltransferase |
| NE1228 | G4 | SAUSA300_0811 | | uncharacterized protein |
| NE1229 | G5 | SAUSA300_1515 | | ABC transporter, permease protein |
| NE1230 | G6 | SAUSA300_0048 | | uncharacterized protein |
| NE1231 | G7 | SAUSA300_0430 | | uncharacterized protein |
| NE1232 | G8 | SAUSA300_2606 | *hisF* | imidazole glycerol phosphate synthase subunit |
| NE1233 | G9 | SAUSA300_0066 | *argR* | arginine repressor |
| NE1234 | G10 | SAUSA300_0112 | *lctP* | L-lactate permease |
| NE1235 | G11 | SAUSA300_0156 | *cap5E* | UDP-galactose 4-epimerase |
| NE1236 | G12 | SAUSA300_0338 | | glyoxalase family protein |
| NE1237 | H1 | SAUSA300_0473 | *purR* | pur operon repressor |
| NE1238 | H2 | SAUSA300_0214 | | uncharacterized protein |
| NE1239 | H3 | SAUSA300_1040 | | uncharacterized protein |
| NE1240 | H4 | SAUSA300_1768 | *lukD* | leukotoxin LukD |

| | | | | |
|---|---|---|---|---|
| NE1241 | H5 | SAUSA300_1222 | *nuc* | nuclease |
| NE1242 | H6 | SAUSA300_1391 | | phiSLT ORF527-like protein |
| NE1243 | H7 | SAUSA300_1398 | | phiSLT ORF123-like protein |
| NE1244 | H8 | SAUSA300_1443 | *rluB* | pseudouridine synthase |
| NE1245 | H9 | SAUSA300_1484 | | uncharacterized protein |
| NE1246 | H10 | SAUSA300_1722 | *sigS* | RNA polymerase sigma factor SigS |
| NE1247 | H11 | SAUSA300_1796 | | UPF0754 membrane protein |
| NE1248 | H12 | SAUSA300_1971 | | phi77 ORF017-like protein |

**Protocol: Transduction of bacterial plasmids into *S. aureus* strains in a 96-well format (e.g. mutant strains of an arrayed transposon library)**

**Materials:**

Bacterial strains and bacteriophages

- Donor *S. aureus* strain carrying reporter plasmid (e.g. bioluminescent reporter genes under control of a promoter of interest)
- Recipient *S. aureus* strains in 96-well format (e.g. subset of, or entire NTML)
- Bacteriophage ϕ11 stock

Media

- Tryptic soy broth (TSB)
- Tryptic soy agar (TSA)
- Soft agar: 0.5% (w/v) agar in TSB
- TSB supplemented with 20% (w/v) glycerol (filter-sterilized)
- Appropriate antibiotics (e.g. to select for transductants that received the reporter plasmid)

Solutions and buffers

- 1M sodium citrate
- 1M $CaCl_2$
- Phage buffer (either filter-sterilized or autoclaved before addition on sterile $CaCl_2$, stored at 4 °C): 50 mM Tris-HCl (pH 7.8), 1 mM $MgSO_4$, 4 mM $CaCl_2$, 90 mM NaCl, 0.1% (w/v) gelatin

Consumables

- 15 mL centrifuge tube (e.g. Corning 430052)
- 0.45 µm filter (e.g. Millipore SLHV033RS)
- Large petri dish (150 mm, e.g. Fisherbrand FB0875714)
- 96-well round clear bottom plate with lid (e.g. Costar 7007)
- 96-well flat clear bottom black plate with lid (e.g. Corning 3603; or Thermo Scientific 165305)
- Plastic wrap to prevent plate to dry out
- Adhesive plate seals to freeze 96-well plate containing final transductants (Thermo Scientific AB-0580)

Misc. equipment

- Spectrophotometer
- 12-channel pipette (alternatively, an 8-channel pipette can be used)
- Plate reader to assess bioluminescence (e.g. BioTek Cytation series)

**Producing phage stock**

**Note:** Various protocols describing how to prepare *S. aureus* phage lysates are available. While we used the method described below for the experiments in this manuscript (adjusted from (1)), a current and detailed protocol was published recently (2).

1) Inoculate 5 mL TSB supplemented with the appropriate antibiotic with the *S. aureus* donor strain.

2) Incubate overnight (37 °C, shaking, 180 rpm).

3) The next morning, measure $OD_{600}$ of *S. aureus* culture using a spectrophotometer.

4) In a 15 mL centrifuge tube, combine 5 mL TSB, 5 mL phage buffer, and bacteria from overnight culture for a final $OD_{600}$ of 0.2.

5) Add 250 µL phage lysate to the 15 mL centrifugal tube.
   **Note 1:** This step is dependent on the sensitivity of strains for infection as well as number of phages contained in the stock. Consequently, the volume might have to be adjusted for phage-resistant strains or lower concentrated phage stocks.
   **Note 2:** Phage lysate stock is generated from WT *S. aureus* using the same method described here.

6) Incubate overnight (static, room temperature).
   **Note:** if culture is not cleared until next morning, an additional incubation at 30 °C (static) can be performed until culture has cleared.

7) When solution is clear, filter sterilize using a 0.45 µm filter and proceed to next steps.
   **Note:** phage lysates can be stored at 4 °C for several months.

**Determine phage titer (adjusted from(2), refer to original publication for further detail)**

1) Generate a dilution series of phage stock to 1e-9 (in phage buffer).

2) Transfer 4.7 mL of liquid (e.g. freshly autoclaved or microwaved) soft agar to 15 mL centrifuge tubes before placing them in a 50 °C water bath. Prepare 8 tubes overall (2 tubes for each of the following dilutions: 1e-6, 1e-7, 1e-8, 1e-9).

3) To each tube add 18 µL of 1M $CaCl_2$, 200 µL of a *S. aureus* JE2 (the parental strain used to generate the NTML) overnight culture, and 100 µL of the corresponding phage dilution (1e-6, 1e-7, 1e-8, 1e-9). Prepare 2 tubes for each dilution.

4) Mix tubes carefully by gentle vortexing.

5) Pour tubes on prewarmed TSA plates.

6) After solidification, incubate plate overnight (37 °C, statically).

7) Count plaques the following day.

**Transduction in 96-well format**

1) Add 200 µL of TSB supplemented with 5 µg $mL^{-1}$ erythromycin (resistance encoded within the transposon used to generate the NTML) to each well of a clear well round bottom 96-well plate.
   **Note:** If not indicated otherwise, all subsequent steps should be performed with a 12-well multichannel pipette.

2) Inoculate the preloaded 96-well plate with bacteria directly from freezer stock (i.e. from one of the 96-well plates that are part of the NTML).

3) Wrap plate in plastic wrapping to prevent evaporation.

4) Incubate the wrapped plate overnight (37 °C, shaking, 180 rpm).

5) The next morning, prepare a TSB-phage-$CaCl_2$ mixture and add to each well of the 96-well round bottom plate

| | **TSB** | **Phage stock*** | **1 M $CaCl_2$** | **Total volume** |
|---|---|---|---|---|
| **For 1 well** | 175 µL | 20 µL | 2.2 µL | 197.2 µL |
| **For 96 wells** (**Note:** prepare 5-10% extra to account for loss during aliquoting) | 16800 µL | 1920 µL | 211.2 µL | 18931.2 µL |

* The amount of phage might be adjusted depending on the titer of the phage stock as well as the susceptibility of the recipient strains. A good starting point is to use 20 uL of undiluted phage stock (prepared as described above), while also determining phage titer for subsequent iterations. It is advisable to use the lowest amount of phage that still generates sufficient transductants carrying antibiotic resistance encoded by the reporter plasmid. For many applications a multiplicity of infection (MOI, # phage/# bacteria) of 0.1 is suited.

6) Transfer 25 µL from each well of the overnight plate into each corresponding well of the newly prepared plate containing the TSB-phage-$CaCl_2$ mixture.

7) Incubate for 30 min (37 °C, statically).

8) Add 6.7 µL of 1 M sodium citrate into each well.

9) Pellet bacteria by centrifugation (4,000 *x g*, 10 min) and resuspend in 200 µL TSB supplemented with 5 mM sodium citrate.
   **Note:** For this as well as subsequent steps, TSA and TSB containing 5 mM sodium citrate should be prepared freshly (e.g. weekly) as this reduces the chances for phage contamination of generated transductants.

10) Incubate for 1 h (37 °C, shaking, 180 rpm)

11) Spot-plate 10 µL of each well on a 150 mm plate TSA supplemented with i) 5 mM sodium citrate and ii) antibiotic for selection of reporter plasmid.
    **Note:** This step is dependent on the transduction efficiency. The goal is to obtain isolated colonies. For an initial trial, additionally plating of a 1:10 can be beneficial.

12) Incubate plate overnight (37 °C, statically)

13) The next day, carefully patch single colonies from each of the 96 spots on a fresh 150 mm TSA plate supplemented with i) 5 mM sodium citrate and ii) antibiotic to ensure maintenance of reporter plasmid.
**Note 1:** Importantly, this step is the only one within this workflow that should be performed without the use of a multi-channel pipette to isolate individual colonies and avoid working with mixed cultures. Too many colonies in each spot can prevent the transfer of individual colonies. In such case, a 1:10 dilution can be used (see step 11) and/or the amount of phage for the initial transduction reduced.
**Note 2:** During manual plating, a grid layout matching 96-well format should be used to ensure compatibility with the use of a multi-channel pipette for subsequent steps.

14) Incubate TSA plate overnight (37 °C, statically)

15) The next day, add 200 µL of TSB (supplemented with antibiotic to ensure maintenance of reporter plasmid) to each well of a new 96-well round bottom plate.

16) Using a multi-channel pipette, transfer bacteria from the TSA plate into each corresponding well of the freshly prepared 96-well round bottom plate containing TSB.

17) Wrap plate in plastic wrap to prevent evaporation

18) Incubate the wrapped plate overnight (37 °C, shaking, 180 rpm).
**Note:** If plates were inoculated early in the morning and growth for each well is visible at the end of the day (after ~12 h), the next step can be performed on the same day.

19) Pellet bacteria by centrifugation (4,000 *x g*, 10 min) and resuspend by adding 150 µL of TSB supplemented with 20% glycerol in TSB to each well.

20) Seal plate with cold seals and store at -80 °C.

21) For determination of reporter gene activity, a fresh 96-well plate containing 200 µL of TSB (supplemented with antibiotic to ensure maintenance of reporter plasmid) can be inoculated directly from the frozen stock. After incubation (e.g. overnight +/- additional back dilutions), a black clear bottom 96-well plate containing media of choice (supplemented with antibiotic to ensure plasmid maintenance) can be inoculated and bioluminescence monitored in a plate reader.

**References:**

1. Novick RP. Genetic systems in staphylococci. Methods Enzymol. 1991;204:587-636.
2. Krausz KL, Bose JL. Bacteriophage Transduction in *Staphylococcus aureus*: Broth-Based Method. Methods Mol Biol. 2016;1373:63-8.