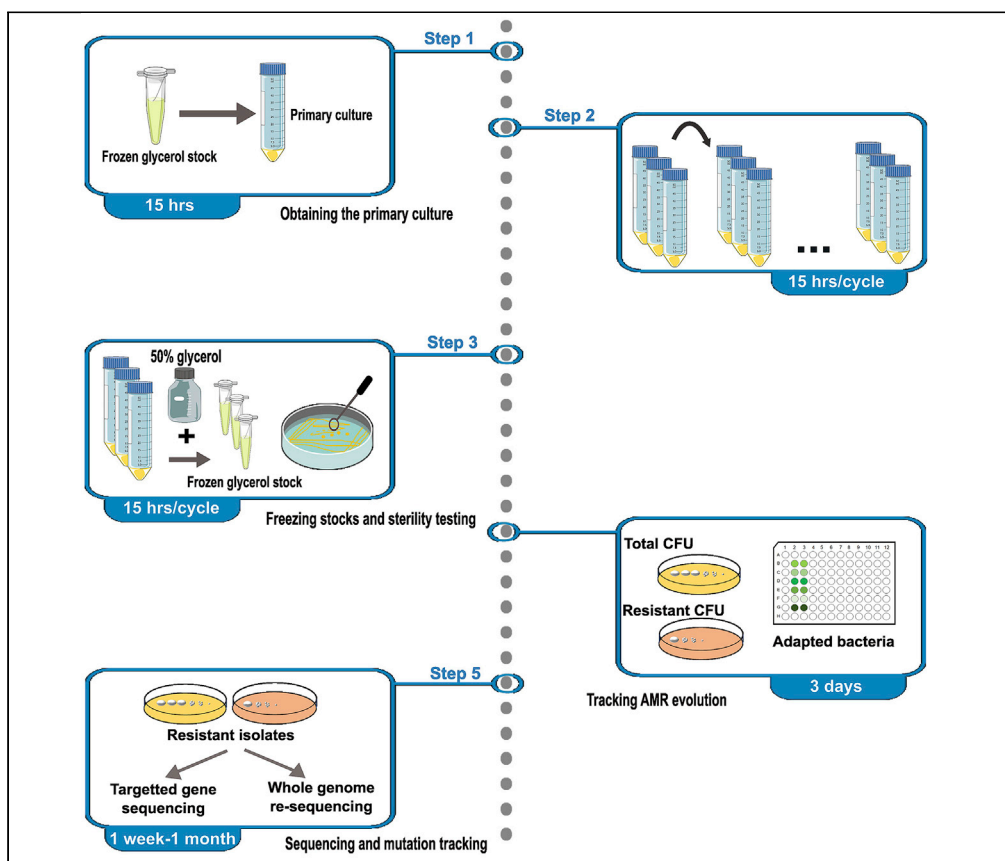# STAR Protocols

Protocol

Adaptive laboratory evolution of antimicrobial resistance in bacteria for genetic and phenotypic analyses

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Highlights

Experimental design principles for laboratory evolution of antimicrobial resistance

Tracking the progress of ALE using titers of resistant bacteria

Rapid protocol for measuring changes in fitness of resistant bacteria

Genetic and genomic analysis of resistant bacteria

Adaptive laboratory evolution (ALE) of bacteria has the potential to provide many insights like revealing novel mechanisms of resistance and elucidating the impact of drug combinations and concentrations on AMR evolution. Here, we describe a step-by-step ALE protocol for the model bacterium *Escherichia coli* that can be easily adapted to answer questions related to evolution and genetics of AMR in diverse bacteria. Key issues to consider when designing ALE experiments as well as some downstream mutation mapping analyses are described.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Protocol

Adaptive laboratory evolution of antimicrobial resistance in bacteria for genetic and phenotypic analyses

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SUMMARY

Adaptive laboratory evolution (ALE) of bacteria has the potential to provide many insights like revealing novel mechanisms of resistance and elucidating the impact of drug combinations and concentrations on AMR evolution. Here, we describe a step-by-step ALE protocol for the model bacterium *Escherichia coli* that can be easily adapted to answer questions related to evolution and genetics of AMR in diverse bacteria. Key issues to consider when designing ALE experiments as well as some downstream mutation mapping analyses are described.

For complete details on the use and execution of this protocol, please refer to Patel and Matange (2021)[1] and Matange et al. (2019).[2]

BEFORE YOU BEGIN

The following protocol describes adaptive laboratory evolution (ALE) of antimicrobial resistance (AMR) in *Escherichia coli* K-12 MG1655 by serially passaging the strain in growth media supplemented with antibiotics. This protocol uses Luria-Bertani broth (LB) and Luria-Bertani agar (LA) as the base media for all experiments. We have used this protocol for successfully evolving resistance against trimethoprim, rifampicin, nalidixic acid, spectinomycin and amoxicillin. However, the protocol can be easily modified for other antibiotics, different growth media and even different bacterial strains with minor changes.

Prepare bacterial growth media and antibiotic stock

⏱ Timing: 4–5 h

1. Prepare required volumes of growth media such as LB or LA by dissolving appropriate quantity of dehydrated media in deionized water.

   Note: Adjust the pH according to the manufacturer's instructions.

2. Sterilize by autoclaving at 121°C for 20 min at 15 psi and allow to cool to 25°C before use.

   Note: Prepared media can be stored at 25°C for at least 1–2 weeks.

3. Make stock solutions of antibiotics in appropriate solvents.

Note: Table 1 lists some commonly used antibiotics, recommended solvents and routinely used stock solution concentrations.

4. Sterilize antibiotic stocks by filtering through a 0.22 μm pore size syringe filter if required and store in aliquots at −20°C.

Table 1. Some commonly used antibiotic stock concentrations and solvents

Antibiotic	Type	Solvent	Stock concentration (mg/mL)
Amikacin	**Amikacin** sulfate	NFW	10
Amoxicillin	**Amoxicillin**	DMSO:NFW (1:1, DMSO: 100%)	10
Ampicillin	**Ampicillin** Sodium salt	NFW	100
Carbenicillin	**Carbenicillin** disodium salt	NFW	50
Cephalexin	**Cephalexin**	1 M Tris-HCl (pH 7.4)	10
Chloramphenicol	**Chloramphenicol**	Ethanol (~95%)	25
Colistin	**Colistin** sodium methanesulphonate	NFW	10
Erythromycin	**Erythromycin**	Ethanol (~95%)	100
Gentamycin	**Gentamycin** sulfate	NFW	10
Kanamycin	**Kanamycin** monosulphate	NFW	25
Nalidixic acid	**Nalidixic acid**, free acid	1 N NaOH	10
Rifampicin	**Rifampicin**	DMSO:NFW (1:1, DMSO: 100%)	10
Spectinomycin	**Spectinomycin** dihydrochloride pentahydrate	NFW	100
Streptomycin	**Streptomycin** sulfate	NFW	10
Tetracycline	**Tetracycline** hydrochloride	Ethanol:NFW (1:1, Ethanol: ~95%)	10
Trimethoprim	**Trimethoprim**	DMSO (100%)	10

NFW: Nuclease-free water; DMSO: Dimethyl sulfoxide.

Prepare the 'ancestral' bacterial stock

⏱ Timing: 3–4 days

Note: We routinely use *Escherichia coli* K-12 MG1655 as the 'ancestral' strain for our experiments and the protocol described here can be directly applied to most enterobacteria. In principle, this protocol can be adapted for any culturable bacterium, with appropriate modifications in growth conditions. However, for ALE experiments to be most effective, we recommend that the bacterial strain of choice should satisfy as many of the criteria listed in Step 5 as possible.

5. Bacterial systems most suited to ALE typically satisfy as many of the following criteria as possible:
   a. Easily culturable under laboratory conditions using readily available growth media.
   b. Relatively fast-growing: Bacteria that can complete a complete growth cycle within 24 h lend themselves most easily to the ALE protocol described here. Most model bacteria such as *E. coli*, *Bacillus subtilis* or *Pseudomonas aeruginosa* are suitable for these experiments.
   c. Genome sequence of bacterium should be available, to allow easy mapping of mutations in evolved isolates.
6. Once the bacterial strain of choice is procured, establishing a clonal population to be used as the ancestor for ALE experiments is essential to ensure reproducibility. For natural/clinical isolates of *Escherichia coli* and similar bacteria, we recommend the following:
   a. Streak out the strain from frozen stock/stab/liquid culture onto an LA plate using a sterile bacteriological loop under aseptic conditions.
   b. Incubate the plate at 37°C for 12–15 h, or until isolated colonies are observed.

c. Pick a single isolated colony using a bacteriological loop or sterile tooth pick and inoculate into 3 mL of LB.
d. Incubate the culture at 37°C with shaking in an orbital shaker-incubator (180–200 rpm) for 12–15 h.
e. Repeat steps a and b at least once more to obtain a clonal population.
f. Aliquot the culture of the purified strain into sterile 1.5 mL microfuge tubes and mix 1:1 with sterile 50% glycerol.

Note: This stock can be stored frozen at −80°C and should be designated as the 'ancestral stock' for ALE experiments.

Note: Ensuring that all Bio-safety protocols are followed and Bio-safety clearances are available is crucial before initiating the above steps. Check for the recommended Bio-safety level of the specific strain of bacterium chosen for the ALE experiments.

When isolating colonies to establish a clonal ancestor, it should be noted that at each step that a single colony is picked for regrowth, a stringent population bottleneck is introduced. This can bring in a number of SNPs into ancestral population that can affect the overall outcome of the evolution experiment. Thus, when using model laboratory strains of bacteria, minimizing the number of culturing steps before starting the ALE is advisable. This will prevent the inadvertent introduction of unwanted mutations.

Designing schema for ALE

⊙ Timing: 5–6 days

7. Antibiotic choice and concentration:

Note: Antibiotic concentration directly alters the strength of selection that mutant bacteria experience in an ALE experiment and hence influences the qualitative and quantitative outcome of the experiment. Typically, there are 2 concentration regimes that are commonly used for ALE, i.e., sub-MIC (minimum inhibitory concentration)[1–4] and dynamically increasing concentrations.[5,6] In our studies we have used sub-MIC concentrations of antibiotics to enrich drug-resistant mutants.[1–3] The sub-protocol below should be appropriately adapted for the specific antibiotic/antibiotic-combination under investigation.

a. Determine the minimum inhibitory concentration (MIC) of the antibiotic for the ancestral strain (Figure 1).
   i. Dilute stock solutions of the antibiotic in the appropriate solvent (refer to Table 1) to obtain a working stock that is 50–100× greater than the highest concentration of the antibiotic to be tested.
   ii. Store the above stock solutions at −20°C until needed.
   iii. Inoculate 10 μL of frozen stock of ancestral strain into 3 mL of LB, and allow growth for 12–15 h at 37°C with shaking at 180–200 rpm to obtain a saturated culture.
   iv. In a sterile, flat-bottom polystyrene 96-well plate, make 2-fold serial dilutions of the antibiotic, using sterile LB as the diluent and the working stock of the antibiotic prepared in (i). Maintain a volume of 100–150 μL in each well.

Note: Use reported values of MIC from literature, if available, as a reference such that the expected MIC is roughly the median value of the range of concentrations used. E.g., For trimethoprim, we routinely use 0.1–50 μg/mL concentrations with the MIC being ∼1 μg/mL.
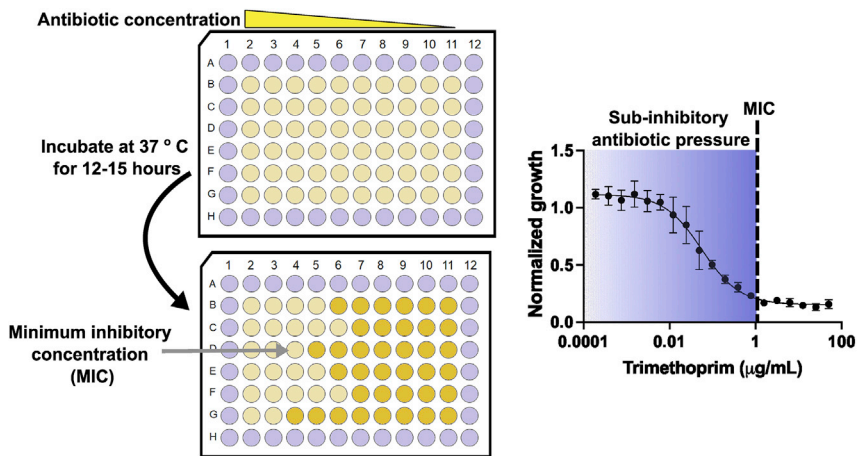
Figure 1. Calculating minimum inhibitory concentration of the ancestral strain against the antibiotic to be used for ALE using broth dilution method in a microtitre plate

Left panel: Diagrammatic representation of the plate set-up needed to calculate MIC. MIC can be defined as the lowest concentration showing no visible bacterial growth or the concentration needed to reduce growth by 90% (MIC90). Right panel: Example data for growth of *E. coli* K-12 MG1655 in different concentrations of trimethoprim. Growth at each concentration was normalized to growth in the drug-free control well. The MIC and sub-inhibitory antibiotic concentrations are indicated. Mean ± S.D. from three independent replicates are plotted.

    v.   Keep an antibiotic-free control well containing LB.

    vi.   If the antibiotic stock is prepared in a solvent other than water, maintain a solvent control to check the effects of solvent alone, if any, on bacterial growth.

    vii.   Inoculate 1 μL of the saturated culture of the ancestral strain into each well.

    viii. Fill the peripheral wells of the 96-well plate with sterile water to prevent dehydration.

    ix.   Incubate the plate at 37°C for 12–15 h in an incubator, with shaking at 180–210 rpm.

    x.   Measure the optical density at 600 nm for all the wells in a 96-well plate reader and normalize growth at each concentration to the antibiotic-free control.

    xi.   MIC can be defined as the lowest concentration of antibiotic that shows at least 90% growth inhibition (i.e., MIC90). E.g., for trimethoprim, *E. coli* K-12 MG1655 shows MIC values ∼1 μg/mL.

    xii.   Perform the above measurements for at least 3 biological replicates, i.e., at least 3 independent cultures of the same bacteria strain and use the average value of MIC for further experiments.

b. Selecting the concentration of antibiotic for ALE:

Note: MIC serves as a reference to decide the concentration of antibiotic to be used for ALE. If concentrations close to or higher than MIC are used, it could lead to sterilization of the culture before the evolution of drug-resistant mutants. On the other hand, if very low concentrations are used there may not be sufficient selection pressure to select for resistance.

    i.   A concentration of MIC/3 typically serves as sufficient pressure for ALE of drug-resistant bacteria, though concentrations as low as MIC/20 can also be used. e.g., For trimethoprim, we have used concentrations ranging from 50 ng/mL (MIC/20) to 300 ng/mL (∼MIC/3) for ALE experiments.[1,3]

    ii.   When using a dynamically increasing concentration regime, a 2-fold increase in antibiotic concentration at regular intervals (e.g., every 25–30 generations of growth) serves to preferentially select for high-level resistant mutants.

    iii.   Alternative to ii, population MIC values can be calculated periodically, and concentration of antibiotic adjusted accordingly.
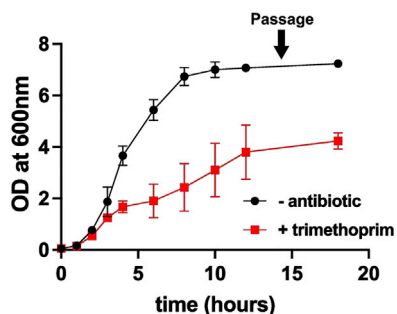
Figure 2. Growth characteristics of the ancestral strain
An illustrative growth curve of the ancestral strain in the presence of 0.25 × MIC trimethoprim (red) and in drug-free growth media (-antibiotic; black). The optimal time point for passaging between cycles of growth in the ALE is indicated. At each time point, mean ± SD from 3 replicate cultures are plotted.

Note: For *E. coli*, we routinely use LB medium for ALE experiments and hence calculate all MIC values in LB medium. If other kinds of media are to be used, make sure to measure the specific drug MIC in each of the media as changes in constituents of growth media can significantly alter antibiotic susceptibility.

Note: If drug combinations are to be employed during the ALE experiment, using a checkboard growth assay[7] can help identify the concentration of the drug-combination to be used.

8. Duration per growth cycle of ALE:

Note: For *E. coli* K-12 MG1655 growing in LB, we routinely allow the culture to grow for 8–14 h before passaging into fresh media.[1] We have also used 24-hour growth cycles with similar success.[2] A 24-hour growth cycle significantly enhances ease of the experiment and avoids having to passage cultures at odd working times. However, it also results in long incubation in the stationary phase. The choice of the length of each growth cycle is thus to be determined taking into account all these factors. Characterising the growth rate and time to saturation can, thus, be helpful prior to starting the ALE experiment. This ensures that in each growth cycle the bacterial cultures grow to saturation and long incubation times in stationary phase are avoided. Following steps can be used to characterise the growth of the ancestral strain in the appropriate medium:

a. Inoculate 10 μL of frozen stock of the ancestral strain into 3 mL of LB (or appropriate growth medium) and incubate for 12–15 h at 37°C with shaking (180–200 rpm) to obtain a saturated primary culture.
b. Inoculate 50 μL of primary culture into fresh sterile growth media (5 mL) as well as growth media supplemented with the desired antibiotic concentration.

Note: Record the time of inoculation.

c. Allow the culture to grow at 37°C with shaking (180–200 rpm).
d. Remove an aliquot of the culture initially every hour, and later every 2–3 h, and measure optical density (at 600 nm) spectrophotometrically.
e. Plot optical density vs time to estimate growth and saturation phases in the absence and presence of antibiotic (Figure 2).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
Escherichia coli K-12 MG1655	ATCC	Cat#700926
Escherichia coli-GFP	Kind gift from Dr. Amrita Hazra (IISER, Pune)[8]	N/A

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Antibiotics (various)	HiMedia/Sigma	–
Trimethoprim	Sigma	Cat#T7883
Miller Luria Bertani Broth, Granulated	HiMedia	Cat#GM1245-500G
Miller Luria Bertani Agar, Granulated	HiMedia	Cat#GM1151-500G
Glycerol	Invitrogen	Cat#15514-011
DMSO	Sigma	Cat#D8418
Ethanol	Sigma	Cat#1009832511
Tris-HCl buffer	MP Bio (Tris base), Qualigens (HCl)	Cat#103133 Cat#Q29145
Gram's staining kit	HiMedia	Cat#K001-1KT
SDS	Merck	Cat#8170341000
EDTA	Sigma	Cat#E9884
Phenol:chloroform:isoamyl alcohol (25:24:1 v/v)	Sigma	Cat#77617
RNase A	Sigma	Cat#10109142001
Proteinase K	Sigma	Cat#1073930010
PrimeStar Max DNA Polymerase	Takara	Cat#R045B
Other		
96-well microtiter plates	HiMedia	Cat#TPG96
1.5 mL tubes	Tarsons	Cat#500010
50 mL graduated centrifuge tubes, PP	Tarsons	Cat#546041
Sterile disposable Petri plates	HiMedia	Cat#PW002
Glass tubes (25 × 150 mm)	Borosil	Cat#9800U08
Bacteriological loops	HiMedia	Cat#LA014
Single use syringes (10 mL)	BD Life Science	Cat#309110
Puradisc 25 syringe filters	Whatman	Cat#6780-2502
96-well plate reader	EnSight multi-mode plate reader (PerkinElmer)	Cat#HH34000000
Shaker-incubator	New Brunswick Innova 42	Cat#M1335-0012
MiniSpin mini centrifuge	Eppendorf	Cat#5452000018

STEP-BY-STEP METHOD DETAILS

Obtaining a primary culture for the ancestral strain

⏲ Timing: 12–15 h

1. Partially thaw a vial of frozen glycerol stock on ice.
2. Inoculate 10 μL of the frozen glycerol stock of the 'ancestral' strain using a micropipette in 3 mL of drug-free LB broth.
3. Incubate for 12–15 h at 37°C with shaking at 180–200 rpm.

Note: The above steps yield a saturated primary culture that will serve as the ancestral bacterial population for setting up replicate evolving bacterial populations.

Note: It is desirable to set up different replicate evolving populations starting from the same primary culture. This ensures that all replicate evolving lineages start from a common ancestral genotype. If mutation mapping using genome sequencing is planned, it is also desirable to sequence this ancestral population so as to filter out pre-existing mutations.

Adaptive laboratory evolution

© Timing: 12–15 h per growth cycle

ALE of AMR is facilitated here using a serial passaging protocol involving repeated growth cycles of bacteria in the appropriate concentration of antibiotic to establish 'lineages' of evolving bacterial populations. Each lineage represents a single replicate of the ALE experiment. The following protocol is described for triplicate lineages of *E. coli* grown in culture volumes of 3 mL, with a 1% bottleneck at each passage. The protocol can be easily adapted for different replicate numbers, culture volumes and bottlenecks (see notes below).

4. Using the primary culture, inoculate 30 μL into 3 mL of growth media with antibiotic at appropriate concentration in triplicate.
5. Label the above as lineages A-C.
6. In parallel, inoculate 30 μL into 3 mL of growth media without antibiotic to establish lineages D-F.

   Note: These control lineages serve to identify changes specifically due to antibiotic exposure.

7. Incubate cultures at 37°C for 12–15 h (or for determined amount of time) with shaking at 180–200 rpm.
8. The above culture represents the first growth cycle (or passage 1) of the ALE experiment. Use this culture to reinoculated into fresh media with antibiotic added at the appropriate concentration to initiate growth cycle 2 (or passage 2).
9. Repeat for as many cycles of growth as needed (see notes below).

   Note: When planning for ALE Steps 4-9 the following parameters should be carefully considered:

Scale of evolution experiment:

The number of replicates and volume of culture per replicate are important parameters to consider when performing an ALE experiment. When a large number of replicates are desirable (>10) using small culture volumes of 150–200 μL in 96-well plates is most convenient. Typically, mutation rates of 1 in $10^{8/9}$ are observed for most antibiotic resistant mutants, which limits the number of mutants in each replicate to 1 or 2 for ~200 μL cultures. If a greater number of mutations are desirable (say, when the effect of competition between different mutants is to be assessed) we recommend using culture volumes of 1 mL or more. In this case, using sterile 50 mL centrifuge tubes or glass tubes ensures sufficient aeration of the culture and convenient handling. Ensure that at least 3 replicate evolving lineages are propagated to test for reproducibility of the ALE.

Duration and number of generations of evolution:

The number of generations for which the evolution experiment is to be performed strongly influences the outcome of ALE experiments. A standard ALE experiment for isolating drug-resistant bacteria can be performed for 20–100 generations, though longer experiments may be required to answer specific types of questions. Two parameters, i.e., passage bottleneck and number of passages of ALE, can be conveniently adjusted in order to achieve the desired number of generations of evolution. Passage bottleneck of 0.1%–10% can be used for the ALE experiment, though 1% is most commonly employed which results in 6–7 generations per growth cycle. To calculate number of generations with each growth cycle use the following formula:

$$2^g = dil$$

Where *g*: number of generations per growth cycle.

dil: dilution factor at each passage (i.e., 100 for 1% passage).

Freezing of evolving lineages

⏱ Timing: 10–15 min at each growth cycle

Freezing the evolving lineages intermittently ensures that a 'fossil record' of the various stages of AMR evolution are preserved. This step is useful to trace the genetic or phenotypic trajectories that resistant bacteria took to evolve. Frozen stocks are also useful for more practical reasons since they can be used to re-initiate the ALE experiment from an intermediate time point in case of contamination, thus saving time. We routinely freeze populations every 10 passages (∼60–70 generations) for long ALE experiments and more frequently for shorter duration experiments in addition to the final evolved population at the end of the ALE experiment.

10. Add 500 μL of the evolved culture at the desired time point to be frozen in a sterile 1.5 mL microfuge tube.
11. To it, add 500 μL sterile 50% glycerol.
12. Gently mix by pipetting until homogeneous.
13. Label the tube with passage number/number of generations, antibiotic and lineage name (i.e., A-F).
14. Store at −80°C.

Checking for contamination

⏱ Timing: 15–20 h

Testing for contamination is crucial at regular intervals, for example before freezing, as it ensures purity of the culture. Contaminating microorganisms, usually other bacteria or yeast can take over the evolving population or affect the evolution of the test organism, particularly during long-term ALE experiments. If contamination is detected, it is advisable to re-start the ALE experiment from the last 'clean' frozen stock. Following are some of the possible quality control steps that may be employed at regular intervals during the ALE experiment.

15. Quality control steps to check for contamination:
    a. Visual cues: Alteration in color or turbidity of growth media can be visible to the naked eye and may indicate contamination. If such signs are observed, test the purity of the evolved cultures using steps b and c.
    b. Microscopic examination: Perform Gram's staining for the evolving populations, and ensure the expected Gram characteristics and morphology is observed.
    c. Colony characteristics:
       i. Dip a nichrome loop in the culture/frozen stock to be tested for contamination and streak out onto the surface of an antibiotic-free LA plate.
       ii. Incubate the plate at 37°C for 15–20 h.
       iii. Note any deviations from the expected colony characteristics, for example, alterations in colony color, size, margin, etc.

Note: Changes in colony characteristics, in particular size, can be expected even for uncontaminated cultures as resistant bacteria may often have different growth characteristics than the ancestor. If a change in colony characteristics or heterogenous colony morphologies are observed, it is advisable to perform a Gram stain to distinguish between contaminants and evolved bacteria.
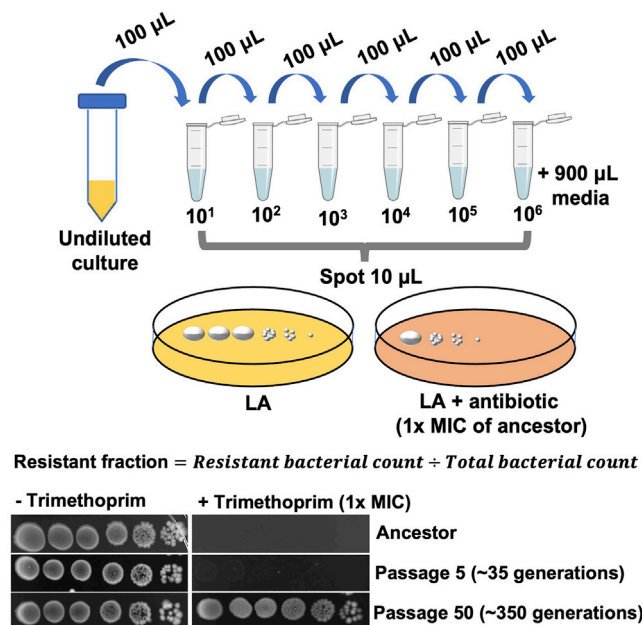
Figure 3. Tracking the evolution of resistance by enumerating the titers of antibiotic-resistant bacteria

The evolution of resistance in the ALE experiment can be tracked by counting the fraction of resistant bacteria in the population at different time points. A simple colony formation assay (shown diagrammatically) can be used to determine what fraction of the evolving population is phenotypically resistant and can also be used to isolate resistant clones for further characterization. An illustrative example from *E. coli* evolving in sub-inhibitory trimethoprim from 2 different time points is shown. Here, bacterial populations from 35 and 350 generations of evolution were serially diluted and grown on LA (-trimethoprim) and LA (+ trimethoprim at 1× MIC).

Often contamination by the same species of bacteria is also observed and can usually be the result of media, pipettes or other commonly used equipment being contaminated. This kind of contamination will go unnoticed by the methods listed in step 12. Maintaining a 'no-bacteria' control tube at each passage can help identify such kinds of contamination.

Tracking AMR evolution, method 1: Enumeration and isolation of phenotypically resistant bacteria

© Timing: 3 days

Tracking the evolution of resistant bacteria over the progress of the ALE experiment and isolation of resistant clones are critical for any downstream characterization steps, such as genotyping or phenotypic analyses. Quantitative estimates of how quickly resistant bacteria take over the population can also be useful in testing the effects of drug combinations or other environmental factors.

16. Enumeration of resistant bacteria (Figure 3).
    a. Inoculate 10–20 μL of frozen stocks from desired timepoints in 3 mL of LB and allow the culture to grow for 12–15 h at 37°C with shaking at 180–200 rpm.
    b. Dilute the culture 10-fold by mixing 900 μL of sterile LB and 100 μL of culture.
    c. Repeat step b serially until a dilution factor of 10^6 is achieved.
    d. Drop 10 μL of each dilution of the culture on an antibiotic-free LA plate (for total bacterial count) and antibiotic supplemented LA plate (for resistant bacterial count).
    e. Allow the drop to dry with the lid of the plate open.
    f. After the drop has dried, close the plate lid and incubate for 12–15 h at 37°C.
    g. Count the number of colonies from the highest dilution that shows discrete colonies from both plates.

h. Calculate the total bacterial count and resistant bacterial count as <u>C</u>olony <u>F</u>orming <u>U</u>nits (CFU) per mL using the following formulae:

$$Total\ bacterial\ counts\ (CFU\ per\ mL)\ =\ Number\ of\ colonies\ on\ LA\ plate\ x\ 100\ x\ dilution\ factor$$

$$Resistant\ bacterial\ counts\ (CFU\ per\ mL)\ =\ Number\ of\ colonies\ on\ antibiotic\ plate\ x\ 100\ x\ dilution\ factor$$

$$Resistant\ fraction\ =\ Resistant\ bacterial\ count \div Total\ bacterial\ count$$

17. Isolation of resistant bacteria.
    a. From the antibiotic-supplemented plate, pick up the required number of colonies of antibiotic resistant bacteria using a sterile loop or tooth pick into 3 mL LB.
    b. Incubate cultures for 12–15 h at 37°C with shaking at 180–200 rpm.
    c. Store the above pure cultures of isolates from ALE as glycerol stocks at −80°C.

Note: The antibiotic concentration used in growth media for isolating resistant bacteria from evolved lineages should be the lowest concentration needed to inhibit the growth of the drug-sensitive ancestor. We routinely use 1–2× MIC concentration to discriminate between colonies of ancestral (drug-sensitive) and evolved (drug-resistant) bacteria. e.g., for trimethoprim we use 1× MIC (1 μg/mL). Alternatively, clinical breakpoint concentrations of antibiotics can be used to isolate resistant bacteria. While doing so results in greater clinical relevancy, it is possible to miss novel adaptations that confer low-level resistance. We therefore advise choosing an objective-appropriate concentration of antibiotic during this step.

It is advisable to re-streak isolates derived from the ALE experiment in order to purify them before freezing.

Validation of resistant bacteria

⊙ Timing: 3 days

18. Revive resistant isolates derived from the ALE by inoculating 10 μL of frozen stock into 3 mL of LB and grow for 12–15 h at 37°C with shaking at 180–200 rpm.
19. Calculate the MIC of the isolates against the antibiotic used during the ALE experiment as described earlier.

Note: An increase in MIC by at least 2-fold compared to the ancestor can be considered as a resistant phenotype for laboratory strains. However, with increase in the number of generations and/or the concentration of antibiotic used for evolution, the MIC may increase to a level beyond the clinical breakpoint. Using the clinical breakpoint to define resistance evolution may also be considered, depending on the question being addressed in the ALE experiment.

Tracking of AMR evolution: Method 2: Using competitive quotient to track the progress of ALE

⊙ Timing: 3 days

Since adaptation to antibiotics involves a progressive enhancement of competitive fitness relative to the ancestor in the presence of the antibiotic, this property can also be used to evaluate the progress of the ALE experiment. Conventionally relative fitness is calculated using competition with a genetically marked ancestor and enumerating the relative proportions of ancestral and evolved bacteria using colony counts or flow cytometry. We have developed a quicker protocol using a GFP-marked

derivative of the ancestral strain. This method doesn't deliver the relative fitness directly, but can be used to track the competitive fitness of evolving bacteria indirectly by calculating the 'competitive quotient'.[1] Since this procedure doesn't involve colony counting or cell sorting, it can be easily used for comparing a large number of replicates or conditions.

20. Revive the frozen stocks of evolving populations derived from the ALE by inoculating 10 μL into 3 mL of LB. Grow for 12–15 h at 37°C with shaking at 180–200 rpm.
21. In parallel, revive GFP-tagged ancestral strain (*E. coli*-GFP) by inoculating 10 μL of frozen stock into 3 mL of LB and grow for 12–15 h at 37°C with shaking at 180–200 rpm.
22. Into 3 mL LB (± antibiotic at the concentration used during ALE) add 15 μL each of saturated cultures of evolved populations and GFP-tagged ancestor.
23. Mix well by vortexing.
24. As controls set up the following cultures:
    a. Mix 15 μL each of ancestor and GFP-tagged ancestor in 3 mL LB (± antibiotic at the concentration used during ALE).

    Note: This control is used to determine the fitness effects of GFP expression.

    b. Inoculate 30 μL of GFP-tagged ancestor to 3 mL LB (± antibiotic at the concentration used during ALE).

    Note: This control is used to determine effect of antibiotic on GFP fluorescence, if any.

25. Allow mixed cultures to grow at 37°C for 12–15 h with shaking at 180–200 rpm.
26. Aliquot 200 μL of each competition into a 96-well plate (in duplicate for each culture). Also inoculate 200 μL from each of the control tubes.
27. Measure the optical density at 600 nm and GFP fluorescence in a 96-well plate reader.
28. Dilute the culture as needed to ensure that values of GFP and optical density are within the range of the plate reader used.
29. Normalize all GFP values by dividing the value of raw fluorescence with OD at 600 nm (nGFP).
30. Calculate competitive quotient using the following formula:

$$competitive\ quotient = \frac{[\{nGFP(c) - nGFP(t)\} - \{nGFP(c) - nGFP(wt)\}]}{[nGFP(c) - nGFP(wt)]}$$

where, nGFP(c), nGFP(t) and nGFP(wt) are the normalized GFP fluorescence values of *E. coli*-GFP alone, mixed culture of *E. coli*-GFP and test strain, and mixed culture of *E. coli*-GFP and ancestor respectively.

31. Compare competitive quotient for different number of generations under antibiotic selection in LB + antibiotic to assess adaptation to the antibiotic and antibiotic-free LB to assess the fitness cost of adaptation (Figure 4).

Mutation mapping in evolved isolates: Method 1: Targeted sequencing of resistance-associate genetic loci

⏱ **Timing: 1–2 weeks**

Antibiotic-resistant mutants isolated in step 13 can be further analyzed to identify resistance-conferring mutations. If specific gene loci are expected to be implicated, a targeted sequencing approach can be used. However, if no information regarding expected mutations is available or if novel genetic determinants of AMR are to be investigated, unbiased whole genome sequencing approaches
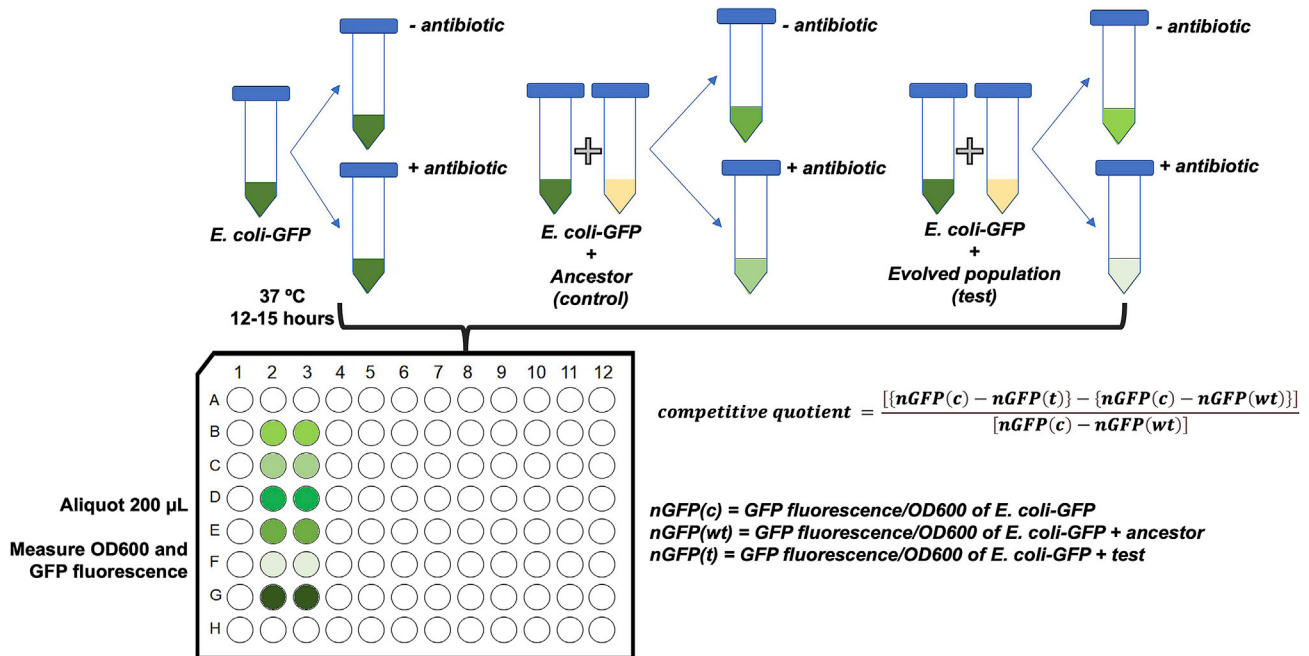
Figure 4. Tracking of the evolution of resistance by calculating the competitive quotient using a GFP-expressing ancestor
The above schematic shows the set-up of a series of competition experiments that can be used to determine the 'competitive quotient' of the evolving populations.[1] This is a quick and simple method for tracking adaptation to antibiotic in the evolving lineages and provides an alternative to CFU or cytometry-based approaches to determine relative fitness.

are required. While the latter are more costly, they can reveal interesting aspects of how adaptation to antibiotics is mediated in bacteria. For bacteria with assembled genomes, this can be a very powerful tool for discovery. Both protocols involve extracting genomic DNA from the resistant isolate as the first step. We routinely use the following for our experiments.

32. Preparation of genomic DNA from drug-resistant isolates and ancestral strain.
    a. Inoculate 10 μL of frozen stock of antibiotic-resistant isolate and ancestor into 3 mL of LB.
    b. Grow for 12–15 h at 37°C with shaking at 180–200 rpm.
    c. Harvest cells in a 1.5 mL microfuge tube by centrifugation and resuspend in 500 μL Tris-EDTA buffer by vortexing vigorously.
    d. Add 25 μL of 10% SDS, mix by inverting the tube and incubate at 37°C for 1 h.
    e. Add 2 μL of Proteinase K (20 mg/mL) to the tube and incubate at 60°C for 3–4 h.
    f. Add 500 μL of phenol: chloroform: isoamyl alcohol (25:24:1) and mix well by inverting.
    g. Centrifuge for 10 min at 13,000 g using a fixed angle rotor in a table top centrifuge.
    h. Transfer upper aqueous layer to a fresh microfuge tube using a wide-bore or cut pipette tip.
    i. Precipitate DNA by adding 0.2 volumes of 3 M Sodium Acetate and 2.5 volumes of chilled 95%–100% ethanol, and gently inverting the tube.

    Note: Genomic DNA will appear as thin white/grey fibres.

    j. If precipitate is not immediately visible, incubate on ice for 5–10 min.
    k. Centrifuge for 2 min at 13,000 g and discard the supernatant.
    l. Wash the pellet with 1 mL of chilled 70% ethanol.
    m. Allow pellet to dry, and resuspend in 50–100 μL of Tris-EDTA buffer.

    Note: Incubate at 37°C for 1 h if the pellet does not dissolve at 25°C.

n. Add 1 μL RNase A (10 mg/mL) and incubate at 37°C for 2 h.

o. Check 2.5–5 μL on a 1% agarose gel and visualize using any intercalating dye.

p. Store genomic DNA at −20°C until further use.

Note: If high purity genomic DNA is required, then the above DNA can be further purified using commercial silica-based spin columns.

33. The above genomic DNA can be used directly as PCR template using gene-specific primers to amplify the region of the interest. For PCR amplification, we routinely use the following parameters:

Reagent	Volume (stock concentration)	PCR program
Gene-specific forward primer	1 μL (10 pmol/μL)	1. 95°C–2 min
Gene-specific reverse primer	1 μL (10 pmol/μL)	2. 95°C–30 s
Genomic DNA template	1 μL (50–250 μg/μL)	3. 45°C–55°C – 30 s
2× PrimeStar Max DNA Polymerase	10 μL	4. 72°C – 5 s/1 kb
Nuclease-free water	7 μL	(Repeat steps 2-4 for 30-35 cycles)
		5. Hold at 4°C

34. PCR products from ancestral and drug-resistant samples can be subjected to Sanger sequencing using the forward and/or reverse primers.

35. Comparison of the sequences from ancestor and drug-resistant isolates can be performed by a simple sequence alignment and will reveal mutations, if any, present in the resistant isolates.

Mutation mapping in evolved isolates: Method 2: Using short-read genome resequencing data for identifying novel mutations

⏱ Timing: Variable, depending on number of sequenced isolates

Illumina-based sequencing has become very accessible and affordable in recent years and is a powerful tool to re-sequence bacterial genomes from ALE experiments. We routinely acquire paired end raw data from sequencing runs as .fastq files from commercial sequencing service providers and use breseq for identifying mutations compared to the ancestor. Breseq is a pipeline that was developed by J.E. Barrick with the aim of analyzing genome re-sequencing data from long-term evolution experiments[9] and is an excellent tool for haploid genomes. Sequencing data of 1–2 GB per sequenced genome provides sufficient depth for most experiments. For well-worked out genomes such as *E. coli*, lower depths of reads can also provide reliable information regarding mutations and can cut down costs significantly. It is crucial to sequence the ancestor used for the ALE along with evolved isolates/population to rule out pre-existing mutations if any. Described below is a step-by-step protocol for using short-read paired-end data from Illumina sequencing to map mutations that may be involved in drug-resistance acquisition.

36. Install breseq according to the developer's instructions, taking care to install breseq and dependencies appropriate to the operating system of the workstation.

Note: Extensive documentation regarding installation and running breseq can be found on the Barrick lab webpage.

37. Download an assembled and annotated reference genome from any publicly accessible genome sequence repository.

Note: For *E. coli* K-12 MG166 strain, we use U00096.3 (GenBank) as the reference for all subsequent analyses. Using the most recent assembly lowers the chance of false positives in the analysis.

38. Run breseq for sequencing reads from ancestral genome against the reference genome downloaded in step 31.

Note: This run should take 45–90 min, depending on the size of the data and processing capability of the workstation used. Breseq generates an easy to navigate output as an .html file that can be opened in most browsers. It also generates outputs as .txt files that are easier to work with for archiving or searching.

39. Run breseq for sequencing reads from evolved isolates against the same reference genome as used in 32.
40. Identify all genomic changes relative to the output that are predicted in the evolved isolate, but absent from the ancestor. Following categories of mutations are possible to identify using breseq:
    a. Gene duplication/amplification: Large gene duplication and/or amplification events can be inferred based on coverage depth plots.

Note: Since bacterial genomes are haploid, the ancestral genome should have roughly the same read depth distribution across all genomic positions. However, bacterial genomes under antibiotic selection can often undergo amplifications of certain regions, which appear as stretches of higher coverage depth than the rest of the genome.[1] These amplifications are usually mediated by mobilization of IS elements. As a result, confirming the presence of repeat sequences flanking the amplified region can be used as a confirmation of the output from breseq.

    b. Single nucleotide substitutions/deletions/insertions: Single nucleotide substitutions/deletions/insertions are directly predicted by breseq.

Note: The predictions made by breseq can be confirmed using the evidence alignment provided along with the output. Marginal predictions, i.e., those that don't make the statistical cut-off can also be considered, provided the evidence alignments are in agreement with the prediction.

    c. Large deletions/transpositions: Large genomic deletions or transposition events are often predicted as 'unassigned junctions' by breseq.

Note: These predictions are based on the premise that large genomic changes create novel junctions between DNA sequences. Evaluating the relevance of large deletions or transpositions by analysing the evidence alignment is crucial to ensure that the predictions are valid. For validating transpositions events for IS elements, looking for pairs of novel junctions (one for each end of the IS element) can provide confirmatory evidence.

EXPECTED OUTCOMES

The above experimental pipeline results in the evolution of antimicrobial resistant bacteria, derived from an ancestral strain that can be used for further analyses. Downstream analyses include, but are not limited to, mutation mapping using targeted gene sequencing or unbiased whole genome sequencing.[1,2] Resistant isolates derived from ALE experiments can also be used for genotype-phenotype mapping, especially when looking for novel mutations associated with AMR,[1,2,4,10] or when trying to understand the impact of different mutations on drug MICs or organismal fitness.[3]

Depending on the strength of selection, resistant bacteria can be fixed in the evolving lineages as early as 15–20 generations.[2–4,11] Longer term experiments can also give information regarding how the phenotypes of drug-resistant bacteria change with extended periods of antibiotic exposure or the evolution of second site mutations that enhance the level of resistance or fitness of resistant mutants.[1,10,12–14]

Apart from yielding drug-resistant mutants, the ALE protocol described here can also be used to compare the rates and frequencies of evolution of resistant bacteria facing different environmental conditions. For instance, we have used this protocol to compare the impact of different concentrations of trimethoprim on the frequencies of resistant bacteria in populations of *E. coli*.[1,2] In this regard, we have found that the use of resistant bacterial titers and competitive quotients can provide important and complementary information regarding the mechanisms of adaptation to antibiotic. For example, at higher concentrations of trimethoprim the gradual enrichment of resistant bacteria correlated with increase in competitive quotient, at lower concentrations, adaptation to the antibiotic was not necessarily driven by enrichment of resistant mutants. Instead, tolerance was preferred and was the predominant form of adaptation.[1]

LIMITATIONS

One of the main limitations of this protocol is that the ancestral strain is necessarily easily culturable. Since this protocol relies of repeated growth and passaging of bacteria, using a relatively difficult-to-culture bacterial strain significantly lowers the success of this protocol in evolving and isolating drug resistant bacteria.

A second limitation is that the number of replicates possible for an ALE experiment are limited since the protocol involves manual passaging. If a large number of replicates are required, automation of certain steps may be considered.

The third limitation of this protocol is that the kinds of mutants selected in the ALE is strongly dependent on the ALE conditions. The serial passaging ALE protocol described here typically enriches the most frequently occurring and most fit mutants. Likewise, there is a possibility of losing low frequency mutants at each bottleneck. Though this can be partially circumvented by reducing the stringency of the bottleneck, it is not completely avoidable. Thus, if a 'random sample' of resistant mutations are required, it may be more appropriate to derive them from a fluctuation test[15] rather than an ALE experiment.

TROUBLESHOOTING
Problem 1
Crashing of population during ALE (steps 4–9).

Potential solution
If no growth is observed after completion of the incubation period for a passage, it can mean that the population has crashed. This is most likely due to very high concentrations of the antibiotic used for ALE. Restarting the ALE with a lower concentration of the antibiotic can remedy this problem. Also, re-check the calculated MIC for the ancestor to make sure that it was determined accurately.

Problem 2
No resistant colonies/low numbers of resistant colonies detected after ALE (steps 16 and 17).

Potential solution
The lack of resistant colonies after the ALE may indicate that the selection pressure for antibiotic resistance was insufficient to enrich resistant mutants. Restarting the ALE with a higher concentration of antibiotic can remedy this problem.

An alternate possibility could be that the concentration of antibiotic used to isolate resistant colonies is too high. A possible solution is to repeat the assay with a range of antibiotic concentrations, keeping the ancestor as a control at all concentrations of the antibiotic. Pick the lowest concentration of antibiotic at which the evolved populations show higher numbers of colonies than the ancestor.

Problem 3
Contamination in ALE cultures (step 15).

Potential solution
Sterilize all media and glassware by autoclaving. Maintain aseptic conditions during inoculation and passaging. Ensure that the evolving cultures are not exposed to contaminants during incubation. If this does not solve the problem, it may indicate that the ancestral strain was contaminated. Purifying the ancestral strain by streaking it out onto growth media and re-establishing a fresh culture from single colony is desirable.

Maintaining a no-bacteria tube at each passage of the ALE may also help identify the source of contamination. Ideally, the no-bacteria control tube should remain sterile. However, if media or any other equipment are the source of contamination, then this tube will show growth.

Problem 4
Degradation of the antibiotic (steps 1–4).

Potential solution
The stability of various antibiotics differs significantly during storage. Loss in stability of the antibiotic can be suspected when large changes in MIC values of the ancestor are observed across replicates. When choosing the antibiotic for ALE, it is recommended to research the properties of the antibiotic of interest before starting the experiment. For instance, many beta-lactams are heat labile.[16] Antibiotics can also react with plasticware, hence reducing the effective concentration of the drug in the medium.[17] These properties can affect efficacy of the antibiotic, and hence the outcome of the ALE experiments. If stability of an antibiotic is doubtful, preparing fresh stock solutions every week or aliquoting the stock solution to prevent multiple cycles of freeze-thaw may be useful.

Problem 5
Too many new mutations detected upon genome sequencing (steps 36–40).

Potential solution
When attempting to identify mutations associated with resistance using genome sequencing, one of the commonly occurring problems is a large number of mutations predicted by breseq. This can make it difficult to distinguish between adaptive and neutral mutations. Under such circumstances one of the following solutions may be considered. Firstly, ensure that the correct reference genome is being used for breseq. Ensure also that all pre-existing mutations (i.e., mutations already present in the ancestor compared to the reference genome) have been filtered. Secondly, obtain sequencing data from control lineages that were passaged in drug-free media. Thirdly, check for possible mutators among resistant isolates. Elevated mutation rates are known to be beneficial when adapting to antibiotics, and are often mediated by mutations in DNA-repair proteins. If mutations are found in known mutator loci then it may explain the large numbers of mutations predicted by breseq.

RESOURCE AVAILABILITY
Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Nishad Matange (nishad@iiserpune.ac.in).

AUTHOR CONTRIBUTIONS

R.V., C.J., and N.M. wrote and prepared figures for this manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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