Modernized tools for streamlined genetic manipulation and comparative study of wild and diverse proteobacterial lineages

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Protocol S2: Allelic exchange with pAX1 and pAX2

Protocol Summary: This protocol describes engineering markerless gene knock-outs with the allelic exchange vectors pAX1 and pAX2. Procedures include the construction of allelic exchange cassettes, delivery of pAX-based vectors into target strains by diparental mating, the selective recovery of merodiploid intermediates, screening for second recombination events, and confirmation of modified target cells. Allelic exchange is a longstanding and widely used technique; therefore, emphasis is placed on using the domestication-free functions of pAX1 and pAX2, merodiploid tracking, and procedural adaptations developed for different bacterial isolates. Also included are common optimization and troubleshooting steps, which are noted within the protocol by the text “OT” followed by a number that references a brief explanation in the “optimization and troubleshooting steps” section of this document. An overview of the procedures is provided in Fig. 3 and the Materials and Methods section of the main text.
**Bacterial strains**

1. **DH5α**: general *E. coli* cloning strain used while constructing allelic exchange cassettes.

2. **Donor**: *E. coli* SM10 carrying a pAX1 or pAX2-based allelic exchange vector.

3. **Target strain**: Bacterial isolate to be modified.
   
   NOTE: The tools developed in this work were designed with genetic parts that are compatible with proteobacterial lineages, especially those belonging to the Gammaproteobacteria. Therefore, vectors may need to be modified with alternative regulatory elements prior to manipulation of more distantly related species.

**Culture media**

1. Lysogeny broth (LB)

2. LB agar plates

3. Tryptic soy agar (TSA) plates

4. Sterile saline wash solution (0.7% NaCl in ddH₂O)
   
   NOTE: The following working concentrations of antibiotics and inducer molecules are added, when needed, to media after autoclaving.
   
   - ampicillin: 100µg/ml
   - gentamicin: 10µg/ml
   - chloramphenicol: 20µg/ml for plasmid maintenance in donor strains and 5µg/ml for selecting modified target cells
   - anhydrotetracycline (aTc): 10ng/ml

**Protocol** (organized by related procedures with the days they are performed indicated)

**Allelic exchange cassette construction**

Below are procedures for constructing an allelic exchange cassette via splice by overlap extension (SOE) (1). To illustrate the procedures, construction of the cassette for generating an in-frame markerless deletion of *pomAB* in *Aeromonas* ZOR0001 is used as an example (Figure 1). The protocol outlined in this section is not meant to be comprehensive; rather, it serves to provide the user with a general overview of techniques involved.
Protocol S2
Allelic exchange with pAX1 and pAX2

I. Primer design and overview of 2-piece SOE
Begin by generating the predicted DNA sequence of the mutant locus using your favorite DNA editing software. Constructing the allelic exchange cassette for markerless deletion of *pomAB* is done via a 2-piece SOE reaction (Figure 2). 2 primer pairs (4 total oligos) will be needed. Using *Aeromonas* ZOR0001 genomic DNA as template, primers WP192 and WP193 amplify the 5’ homology region (5’ HR) and primers WP194 and WP195 amplify the 3’ homology region (3’ HR). Homology regions can vary in size, but 800–1,000bp generally works well. After each homology region is amplified, overhanging homologous tails incorporated by WP193 and WP194 are used to splice the two PCR products together, producing the final allelic exchange cassette. The cassette is then ligated into a SmaI cut pAX vector and sequenced.

II. Amplification of the 5’ and 3’ homology regions
PCR (based on NEB Q5 High-Fidelity DNA Polymerase)
+ One 50µl reaction per homology region
+ Annealing temperature: Determined by the NEB TM calculator tool
+ Extension time: 20-30sec/kb
+ 22 cycles

Example PCR reaction recipe
- Template DNA variable
- Primer 1 (10µM) 2.5µl
- Primer 2 (10µM) 2.5µl
- dNTPs (10mM) 1µl
- 5x Q5 Buffer 10µl
- PCR H₂O variable
- Q5 polymerase 0.5µl

Clean and concentrate PCR products
+ Run 5µl of each reaction on a 1% agarose gel to verify amplification.

+ Clean and concentrate the remaining 45µl using a Zymo DNA clean & concentrator kit. Elute DNA with 25µl PCR H₂O.

+ Measure DNA concentration. For the SOE reaction below, you will use ~50ng of each product.
III. Splice by overlap extension

Conditions (based on NEB Q5 High-Fidelity DNA Polymerase)

+ Two 50µL reactions. During SOE, there is no amplification; therefore, running two reactions helps to increase yield.

+ Annealing temperature: Typically, 72°C (when using Q5 polymerase). Annealing temperature is calculated based on the middle primers (i.e., WP193 and WP194), which are often >40bp and complementary to one another.

+ Extension time: 20-30sec/kb (calculated based on largest homology region)

+ 15 cycles

Example SOE reaction recipe (per 50µl reaction)

- 5' HR (50ng) variable
- 3' HR (50ng) variable
- dNTPs (10mM) 1µl
- 5x Q5 Buffer 10µl
- PCR H₂O variable
- Q5 Polymerase 0.5µl

Gel-purify SOE product

+ Run SOE reaction on a 1% agarose gel. SOE reactions often produce a variety of intermediate products, so make sure the full-length product is extracted. Combine gel slices from both 50µl reactions and gel-purify using a Zymoclean gel DNA recovery kit. Elute DNA in 20µl PCR H₂O.

IV. Amplify SOE product

PCR conditions (based on NEB Q5 High-Fidelity DNA Polymerase)

+ Two 50µL reactions. Two reactions are used to increase yield.

+ Annealing temperature: Calculated based on the outer most primers (i.e., WP192 and WP195) using the NEB TM calculator tool.

+ Extension time: 20-30sec/kb (calculated based on the full-length SOE product)

+ 22 cycles

Example PCR reaction recipe (per 50µl reaction)

- SOE product 1µl
- Primer 1 (10µM) 2.5µl
- Primer 2 (10µM) 2.5µl
- dNTPs (10mM) 1µl
- 5x Q5 Buffer 10µl
- PCR H₂O 32.5µl
- Q5 Polymerase 0.5µl
Gel-purify amplified SOE product
+ Run the amplified SOE product on a 1% agarose gel as above. It is still possible for minor products to be generated, so make sure you recover the predicted full-length product. Combine gel slices from both 50μl reactions and gel-purify using a Zymoclean gel DNA recovery kit. Elute DNA in 44μl PCR H2O.

V. Phosphorylate amplified SOE product
+ To the 44μl solution generated above containing the amplified SOE product, add:
  - T4 Ligase Buffer 5μl
  - T4 Polynucleotide Kinase 1μl

+ Incubate for 30 minutes at 37°C

+ Clean and concentrate using a Zymo DNA clean & concentrator kit. Elute DNA with 16μl PCR H2O. This solution now contains the final allelic exchange cassette.

VI. Prepare the pAX vector
+ Digest ~3μg of isolated pAX vector with Smal
  - pAX vector 43μl
  - NEB Cutsmart Buffer 5μl
  - SmaI 2μl
  Incubate at 25°C for >4h

+ Dephosphorylate blunt ends
  - Add 5μl of NEB Antarctic Phosphatase buffer directly to the SmaI digest
  - Add 1μl NEB Antarctic Phosphatase
  Incubate at 37°C for 30min

+ Clean and concentrate using a Zymo DNA clean & concentrator kit. Elute DNA with 20μl PCR H2O.

VII. Ligate allelic exchange cassette into pAX vector and transform into DH5α
+ Ligation reaction recipe
  - pAX vector 1μl
  - allelic exchange cassette 7μl
  - T4 Ligase Buffer 1μl
  - T4 Ligase 1μl
  Incubate at 16°C overnight

+ Transform entire ligation reaction into DH5α, and plate on selective LB agar. Screen clones by diagnostic digest, and sequence confirm the allelic exchange cassette. Once a verified clone is obtained, move the assembled pAX-based vector into E. coli SM10.
Delivery of pAX-based vectors by diparental mating and isolation of mutant target cells

I. Preparation of bacteria (days 1–2)

+Day 1 Start overnight cultures of donor and target strains.

Donor: Culture donor strain carrying pAX-based vector overnight (~16 to 20h) in 5mL LB media at 30°C with shaking and ampicillin to full density (i.e., an optical density at 600nm (OD$_{600}$) of ≥1.0).

Target: Culture conditions will vary. In this work, target strains were cultured overnight in 5mL LB media at the appropriate growth temperature (i.e., 30°C or 37°C) with shaking to full density.

+Day 2 Subculture donor and target strains, and prepare mating reaction.

-Subculture strains

Donor: Subculture 1:50 into 5ml fresh LB media with shaking and ampicillin at 30°C to an OD$_{600}$ of 0.4–0.6 (typically for 2–3h).

Target: Subculture 1:100 into 5ml fresh LB media at the appropriate growth temperature (i.e., 30°C or 37°C) with shaking to an OD$_{600}$ of 0.4–0.6. Outgrowth time depends on the bacterial isolate, but typically takes 1–2h.

-Mix bacteria

1. Mix subcultured donor and target strains 1:1 (750µl each) in a 1.6ml microcentrifuge tube.

2. Wash mating mixture to remove antibiotics. Centrifuge mating mixture at 7,000xg for 2 minutes, remove supernatant by decanting or aspiration, and suspend bacterial pellet in 1ml wash solution by gently pipetting up and down.

3. Repeat wash step as above, but this time suspend the bacterial pellet in 25µl wash solution.

II. Mating (day 2)

-Incubate mating reaction

1. With sterile forceps, place a sterile 25mm-wide 0.45µm filter disc (Millipore, product #HAWP02500) onto the surface of a TSA plate.

2. Pipette the entire 25µl mating mixture onto the surface of the filter disc and allow to dry for 5–10 minutes.

3. Incubate the assembled mating reaction at 30°C for ~5 hours.

OT1 Extending the incubation time of the mating reaction can improve recovery of merodiploid target cells.
### III. Selection and purification of merodiploid target cells (days 2–4)

#### Plate mating reaction on selection media

1. With sterile forceps, transfer the filter disc to a 50ml conical tube containing 1ml wash solution. Dislodge bacteria by pipetting and/or vortexing.

2. Transfer cell suspension to a 1.6ml microcentrifuge tube.

3. For temperature-based counterselection using either pAX1 or pAX2, spread 100µl of the cell suspension on a TSA/gent plate and incubate overnight at 37°C. For kill switch-based counterselection using pAX2, spread 100µl of the cell suspension on a TSA/gent/aTc plate and incubate overnight at 30°C. Temperature and kill switch-based counterselection can be done in combination using pAX2 by spreading 100µl of the cell suspension on a TSA/gent/aTc plate and incubating overnight at 37°C. For both pAX1 and pAX2, chloramphenicol can be used instead of gentamicin.

4. To ensure recovery of rare recombinants, centrifuge the remaining 900µl of the cell suspension at high-speed (≥16,000xg), decant or aspirate supernatant, suspend bacterial pellet in 100µl wash solution, and spread on the appropriate selection media as in step #3 above.

**OT2** Outgrowth of the mating reaction in liquid media prior to plating can improve recovery of merodiploid target cells.

**+Day 3** Isolate and colony-purify putative merodiploid clones.

1. With a fluorescent stereomicroscope, screen plates for colonies of the target strain that are expressing the merodiploid tracker and exhibit uniform fluorescence.

   • **NOTE:** Colonies with cells that did not undergo the first recombination step to insert the allelic exchange vector into the chromosome will typically have plasmid-based fluorescence/resistance and can be identified because they tend to be brighter and display heterogeneous patterns of fluorescence intensity. However, when manipulating bacterial isolates for the first time, empirical determination of strain-specific phenotypes will be necessary.

2. Colony-purify multiple putative merodiploids (≥2) by streaking individually colonies on TSA plates and incubating them overnight at the growth temperature most appropriate for the target strain (i.e., 30°C or 37°C).

   • **NOTE:** This colony-purification step is intentionally done in the absence of antibiotic selection to test the stability of fluorescence. If the allelic exchange vector successfully recombined into the chromosome, then colony fluorescence will be stably maintained. By contrast, if cells within the colony are merely carrying the vector, then it will be lost as the bacteria divide, along with their fluorescent phenotype.
OT3 Potential complications and points of failure: no merodiploid target cells are recovered; too many merodiploid target cells grow; poor SM10 counterselection.

+Day 4 Culture and freeze down putative merodiploid clones.
1. Inspect streaks with a fluorescent stereomicroscope. Pick 2 merodiploid colonies from separate streaks that exhibit stable fluorescence. Culture each one in 5ml LB media with shaking and gentamicin (or chloramphenicol) at the growth temperature most appropriate for the target strain (i.e., 30°C or 37°C) to an OD<sub>600</sub> of 0.4–0.6 (typically 4–6h). One merodiploid clone will serve as the primary strain from which the final mutant is derived, while the other is a backup.

   • NOTE: In this step, colonies are cultured in the presence of antibiotic selection to verify that the cells still carry the resistance marker encoded by the allelic exchange vector and to maintain their merodiploid state prior to freezing.

OT4 Potential complications and points of failure: merodiploid target cells only carry plasmid-based fluorescence or resistance.

2. Prepare a 1.5ml glycerol stock for each merodiploid clone by mixing 750µl of culture with 750µl 50% sterile glycerol. Store at -80°C.

   • NOTE: The glycerol stock can be prepared in various ways so long as the final glycerol concentration is 25%. In addition, our practice is to freeze bacterial strains (wild or modified) when they reach an OD<sub>600</sub> of 0.4–0.6 because it minimizes the time the cells spend growing under laboratory conditions.

3. Subculture the primary merodiploid strain from step #1 above 1:100 in 5ml fresh LB media at the appropriate growth temperature (i.e., 30°C or 37°C) with shaking overnight. Without antibiotic selection, the merodiploid cells are now free to undergo the second recombination. Let the remaining culture from step #1 continue growing overnight. This culture, which still has gentamicin present, will be used to isolate genomic DNA for genotyping later. Genomic DNA can be isolated right away the next day, or 1ml of the culture can be centrifuged at high-speed for 3 minutes, supernatant removed by aspiration, and the bacterial pellet stored at -20°C.

OT5 Screening for second recombination events can sometimes be expedited by using the culture generated in step #1 above for the serial dilutions and plating described under “Day 5” below.

IV. Screening for second recombination events (days 5–7)

+Day 5 Serially dilute and plate merodiploid culture on TSA.

In the morning, make serial dilutions of the primary merodiploid culture (e.g., 50µl into 450µl wash solution) that had grown overnight without antibiotics to obtain a cell density such that when 100µl is spread onto a TSA plate, ~100-200 discrete colonies will form. Prepare 2–4 plates in this way and incubate at the appropriate growth temperature overnight.
Day 6  Select and colony-purify colonies exhibiting loss of the merodiploid tracker. Colony-purify multiple colonies (≥6) exhibiting full or partial loss of the merodiploid tracker by streaking individual colonies on TSA plates and incubating them overnight at the growth temperature most appropriate for the target strain (i.e., 30°C or 37°C).

**OT6** Potential complications and points of failure: No colonies are found that have full or partial loss of the merodiploid tracker.

Day 7  Culture and freeze down putative mutant clones.

1. Inspect streaks with a fluorescent stereomicroscope. Pick at least 6 colonies with no detectable expression of the merodiploid tracker from separate streaks. Culture each one in 5ml LB media with shaking at the growth temperature most appropriate for the target strain (i.e., 30°C or 37°C) to an OD$_{600}$ of 0.4–0.6 (typically 4–6h).

   • **NOTE:** A minimum of 6 colonies should be selected because ~half will harbor the desired mutation, while the other half will be wild-type revertants.

2. Prepare a 1.5ml glycerol stock for each clone by mixing 750µl of culture with 750µl 50% sterile glycerol. Store at -80°C.

3. Allow the cultures (now 4.25ml) to continue growing overnight, they will be used to harvest genomic DNA for genotyping.

V. Genotyping (day 8)

Day 8  Identification of mutants by PCR.

-Harvest genomic DNA

1. For each putative mutant and the merodiploid parent, pellet 1ml of dense culture (OD$_{600}$ ≥1.0) by centrifuging at high-speed (≥16,000xg), remove supernatant by aspiration, and suspend bacterial pellet in 100µl TE-SDS (10mM Tris, 1mM EDTA, 0.5% SDS) by vigorously pipetting while avoiding air bubbles. It is OK if the cells do not completely resuspend.

2. Heat the cell suspension at 90°C for 5 minutes, vortex briefly (~3 seconds), and continue heating at 90°C for an additional 5 minutes.

3. Centrifuge the cell suspension at high-speed (≥16,000xg) for 10 minutes to pellet cell debris.

4. Without disturbing the pellet, prepare a stock of genomic DNA by transferring 4µl of the supernatant to a 1.6ml tube containing 196µl of molecular grade H$_2$O.

   • **NOTE:** There are many alternative ways to isolate genomic DNA. For PCR-based genotyping of merodiploids and putative mutants, we typically use the “dirty” genomic DNA isolation protocol described above. In cases where cleaner preparations of genomic DNA are needed, we use the Wizard Genomic DNA Purification Kit (Promega).
**PCR-based genotyping**

1. For each clone, prepare and run a 20µl PCR reaction using 0.5µl of the genomic DNA prep generated in the steps above as template. In general, use primers that will amplify across the locus being modified such that wild-type and mutant loci produce distinct amplicon sizes. It is important to genotype the merodiploid, as well as the wild-type parent, to confirm PCR conditions favor amplification of both wild-type and mutant loci.

2. Run the PCR reaction on a 1% agarose gel to determine if the correct amplicon sizes are produced.

**OT7** Potential complications and points of failure: absence of correct amplicons; all putative mutants are wild-type revertants.

3. Prior to conducting experiments with newly engineered strains or discarding redundant clones, verify that basic phenotypic traits not expected to be altered by the mutation are preserved.

**OT8** Potential complications and points of failure: mutant target strain exhibits altered physiology.

**Optimization and troubleshooting steps**

**OT1** Extended mating period: Some target strains exhibit low mating efficiencies. This can be due to a variety of reasons; for example, endogenous restriction systems expressed by the target strain or contact-dependent killing of the donor strain by the target strain. We have found that extended mating times can improve the frequency of successful matings. For example, in the case of *Vibrio* ZWU0020, manipulation of this target strain requires an overnight (~16–20h) mating time. To accommodate expansion of bacterial cells on the filter disc after an overnight mating, recover the cells in 400µL wash solution and spread 100µL on four separate TSA selection plates as described under “Plate mating reaction on selection media”.

**OT2** Post-mating outgrowth: For some target strains, an outgrowth period following conjugation is required to recover merodiploid target cells. The reason for this could be due to slow or inefficient expression of the resistance genes or because merodiploids are rare and need to be expanded. We have found that *Vibrio* ZWU0020 requires a post-mating overnight outgrowth. Typically, outgrowths are performed by transferring the entire mating reaction to a 50ml conical tube that contains 10ml of LB or tryptone broth with gentamicin or chloramphenicol, dislodging bacteria, and incubating overnight with shaking. If temperature-based counterselection is being used, the culture is incubated at 37°C. If kill switch-based counterselection is being used, the culture is incubated at 30°C with aTc. If a combination of temperature and kill switch-based counterselection is being used, then the culture is incubated at 37°C with aTc. The following day, cells are plated as described under “Plate mating reaction on selection media” with slight modification. In addition to
plating 100 µL of the outgrowth culture, a 1 and 2ml volume of cells are also harvested and plated to increase the chances of capturing merodiploid target cells.

**OT3** Potential problematic outcomes after plating the mating reaction on selection media:
- **No merodiploid target cells are recovered.** First, attempt the mating again, double checking that all steps are followed and performed correctly. At the same time, consider the compatibility of the target strain with the tools being used. For example, is the antibiotic resistance marker appropriate? Can the regulatory elements be recognized? Are the homology regions within the allelic exchange cassette sufficiently big (i.e., ≥1,000bp)? Is disruption of the locus being targeted lethal? If modified target cells are still not recovered after a second attempt, try the procedural variations described in OT1 and OT2.

- **Too many merodiploid target cells grow, producing a lawn.** It is possible that the mating or recombination efficiency is high, in which case, make serial dilutions of the cell suspension prior to spreading on selection media so that individual colonies can form. If a lawn of target cells forms that do not appear to be modified, consider that the target strain is either naturally resistant to the antibiotic being used or can readily acquire resistance.

- **Poor SM10 counterselection.** If it appears that SM10 donor cells grow unchecked on the selection media, perform the mating again. We have found that this can happen on occasion and surmise that in some mating reactions there are SM10 clones that escape the genetically encoded counterselection systems. If the problem persists, try plating fewer total cells. We have found that when too many cells are plated on selection media, counterselection efficiency decreases regardless of the counterselection system.

**OT4** Target cells carry only plasmid-based fluorescence or resistance: After colony-purification, if it appears that putative merodiploid target cells only carry plasmid-based fluorescence or resistance, repeat the mating, confirming that the correct selection was performed. Additionally, recombination events during allelic exchange are expected to be relatively rare, and can be overcome by screening a larger number of putative modified target colonies or using an allelic exchange cassette with extended homology regions.

**OT5** Expediting the screening of second recombination events: Depending on the recombinogenic potential of the target strain or locus being modified, second recombination events can readily take place within colonies growing on TSA without selection. Therefore, overnight outgrowth of merodiploid cells in LB media may not be required, shortening the protocol by a day.

**OT6** No colonies are found that have full or partial loss of the merodiploid tracker: If no second recombination events are found, first make sure to perform an overnight outgrowth of the merodiploid in LB media without antibiotic selection. If second recombination events are still not found, increase the number of total colonies screened. Also consider extending the homology regions used for the allelic exchange cassette and if the mutation being engineered is potentially lethal. Of note, second recombination events can sometimes be
found as very small patches within merodiploid colonies and thus, closer inspection may be helpful.

Potential problems for PCR-based genotyping:

- **Absence of correct amplicons.** If no product(s) are generated, repeat the PCR and consider common PCR troubleshooting steps (e.g., primer design, switching the DNA polymerase, annealing temperature, extension time, etc.). We have found that genomic DNA preps from different target strains can interfere with PCR reactions. Using a cleaner preparation of genomic DNA may help. If the amplicon(s) are the wrong size, double check the primer design and predicted sequence of the mutant locus. If there is amplification of nonspecific bands that prevents confirmation, consider trying a cleaner prep of genomic DNA, reducing the concentration of template DNA, altering the annealing temperature or cycle number, or designing alternative primers. On occasion, amplification of both wild-type and mutant loci from the merodiploid can be difficult. Specifically, the larger of the two amplicons can be weak or not present. If this problem is encountered, consider optimizing PCR conditions or using an alternative set of primers.

- **All putative mutants are wild-type revertants.** The recombination events during allelic exchange are expected to occur in random order; therefore, half of the putative mutant clones will harbor the desired mutation, whereas the other half will be wild-type revertants. On occasion, only wild-type revertants are recovered—either the frequency of successful allelic exchange is incredibly rare or by chance only revertants were initially isolated. If this occurs, isolate and screen a larger number of clones. If the problem persists, consider extending the homology regions to balance the chances of recombination on either side of the targeted locus, or if the mutation being engineered is lethal.

Mutant target strain unexpectedly exhibits altered physiology: It is possible that mutant target strains will exhibit altered physiology, despite avoiding domestication steps. If this occurs, consider isolating a new clone from either the primary merodiploid stock or the backup merodiploid. We have found on occasion that phenotypic variants can arise simply through sequential passage in laboratory media independent of any genetic modifications we introduce. Additionally, consider if the mutation disrupts normal expression of nearby genes, for example, is the mutated locus within an operon? To exclude the possibility that the unexpected phenotype is in fact due to the mutation introduced, be sure to perform genetic complementation, if possible.
REFERENCES
1. Horton RM, Cai Z, Ho SM, Pease LR. 2013. Gene splicing by overlap extension: tailor-made genes using the polymerase chain reaction. BioTechniques 8(5):528-535 (November 1990). Biotechniques 54:129–33.