Genetic Manipulation of *Klebsiella pneumoniae*

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Klebsiella pneumoniae is a Gram-negative, rod-shaped bacterium commonly found in the human intestine. Although it typically exists as part of the normal flora, it can also cause healthcare-associated infections with severe consequences. Understanding the specific genes responsible for its virulence through genetic manipulation is crucial for potential therapeutic interventions. However, manipulating *K. pneumoniae* presents challenges due to its exopolysaccharide capsule. This article presents a comprehensive collection of protocols designed to facilitate the genetic manipulation of *K. pneumoniae*. By following these protocols, researchers will acquire the necessary skills to prepare electrocompetent cells, utilize electroporation for efficient plasmid DNA introduction, construct isogenic mutants using the λ Red recombinase system, and generate a complementation vector for restoring the phenotypic traits of knockout strains. These protocols provide valuable tools and techniques to navigate the intricacies associated with studying and modifying *K. pneumoniae*. © 2023 The Authors. Current Protocols published by Wiley Periodicals LLC.

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Keywords: genetic manipulation • *Klebsiella pneumoniae* • mutagenesis • plasmid • transformation

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INTRODUCTION

Klebsiella pneumoniae is a significant human pathogen known for causing a wide range of infectious diseases, including pneumonia, urinary tract infections, and bloodstream

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infections (Wang et al., 2020). It is also well known for propelling the dissemination of antimicrobial resistance among *Enterobacteriaceae*, posing a major challenge in the clinical setting (Hu et al., 2021). Genetic manipulation studies of *K. pneumoniae* have gained considerable attention due to their potential to provide valuable insights into the pathogenic determinants and mechanisms of antibiotic resistance in this bacterium. It is important to note that although considerable progress has been made in the genetic manipulation of *K. pneumoniae*, there are still challenges and limitations.

The presence of an exopolysaccharide capsule in *K. pneumoniae* complicates the genetic manipulation of *K. pneumoniae* by reducing transformation efficiencies (Fournet-Fayard et al., 1995). Hypermucoviscous *K. pneumoniae* strains pose a continued challenge due to the high abundance of capsular polysaccharides (CPS) on the cell surface, resulting in low transformation efficiency (Park & Ko, 2023). However, one major hurdle persisting in the genetic manipulation of *K. pneumoniae* is the absence of standardized protocols tailored for various genetic techniques using hypervirulent strains. Overcoming this challenge will involve optimizing existing protocols to effectively address the specific requirements and obstacles associated with hypervirulent strains.

Here, we provide protocols for efficient genetic manipulation procedures using a hypervirulent strain of *K. pneumoniae,* KPPR1 (Broberg et al., 2014; Lawlor et al., 2005; Standiford et al., 1999). The protocols provide the foundational tools necessary to investigate the significance of a gene or genetic element of interest that is altering a phenotype of interest in *K. pneumoniae*. Basic Protocol 1 and Alternate Protocol 1 describe the preparation of electrocompetent *K. pneumoniae* cells. Basic Protocol 2 details the transformation of plasmid DNA into competent cells through electroporation. Basic Protocol 3 describes the use of a plasmid containing the λ Red recombinase system to generate an isogenic mutant strain. Support Protocols 1 and 2 dictate the methods used to validate that a targeted mutation was made with no off-site effects. Basic Protocol 4 describes the usage of FLP recombinase to generate a clean knockout. Finally, Basic Protocol 5 explains the construction of complementation vectors to restore the phenotype of a mutant.

CAUTION: K. pneumoniae is a Biosafety Level 2 pathogen, and the *Escherichia coli* strains used in this protocol are all a Biosafety Level 1 pathogens. Hazards and precautions associated with this protocol include performing electroporation with high-voltage electrical pulses, utilizing liquid nitrogen or dry ice for flash freezing, and maintaining sterile techniques when handling bacterial cultures. Follow all appropriate guidelines and regulations for the use and handling of pathogenic microorganisms.

NOTE: All protocols involving animals must be reviewed and approved by the appropriate Animal Care and Use Committee and must follow regulations for the care and use of laboratory animals. Appropriate informed consent is necessary for obtaining and use of human study material.

BASIC PROTOCOL 1

PREPARING ELECTROCOMPETENT *K. pneumoniae* CELLS

This protocol is adapted and optimized from previously published methodologies to be an efficient method for the preparation of electrocompetent *K. pneumoniae* cells (Bachman et al., 2015). Competent cells are essential for genetic manipulation and transformation procedures (Basic Protocol 2). Electrocompetent cells can take up foreign DNA through electroporation, allowing the introduction of plasmids, gene knockouts, or other genetic modifications. In brief, this protocol provides step-by-step instructions for the growth of *K. pneumoniae* to mid-exponential phase, preparation of competent cells, and storage of the electrocompetent cells. The resulting electrocompetent *K. pneumoniae* cells can be used in various molecular biology applications.

Materials

Frozen glycerol stock of *K. pneumoniae* reference strains ATCC 43816 (KPPR1) or other strain(s) of interest
Low-salt LB agar (see recipe)
Low-salt LB broth (see recipe)
0.5 mM EDTA, pH 8 (see recipe)
1 mM HEPES, pH 7.4 (see recipe), ice cold, sterile
Sterile ultrapure water, ice cold
10% (v/v) glycerol, ice cold, sterile
Liquid nitrogen

Static incubator (e.g., Thermo Scientific, Heratherm), 30°C
17 × 100-mm aeration culture tubes (Genesee Scientific, cat. no. 21-131)
Shaking incubator (e.g., New Brunswick Innova 44), 37°C
125-ml Erlenmeyer flask (Fisher Scientific, cat. no. 10-040D)
1.7-ml disposable cuvettes (Fisher Scientific, cat. no. 14-955-127)
Spectrophotometer (e.g., Thermo Fisher GENESYS 30)
Ice bucket with ice
50-ml conical tubes (USA Scientific, cat. no. 5622-7261)
Refrigerated centrifuge (e.g., Eppendorf centrifuge 5425R)
1.7-ml sterile microcentrifuge tubes (Genesee Scientific, cat. No. 24-282C)
−80°C freezer

Culture and growth conditions for mid-log phase

1. Streak *K. pneumoniae* strain KPPR1 from the −80°C glycerol stock onto a low-salt LB agar plate and incubate overnight at 30°C.

2. Select a single colony of KPPR1 and inoculate into two culture tubes containing 3 ml of low-salt LB broth and 0.5 µM EDTA. Incubate the culture overnight at 37°C with shaking (200 rpm).

   For other strains of interest requiring antibiotics for growth, add the appropriate antibiotics to the culture tubes.

3. Prepare a sterile 125-ml flask for the next day by adding 50 ml low-salt LB broth and place it in the 37°C incubator overnight.

   Prewarming the low-salt LB broth expedites reaching mid-log phase compared to using room-temperature broth.

4. Aseptically remove 1 ml from the flask of low-salt LB broth and pipet it into a cuvette to serve as a blank for step 6.

5. Inoculate the sterile 125-ml flask containing 50 ml low-salt LB broth with 0.5 µM EDTA and antibiotics, if required. Swirl to mix, add 0.5 ml of the overnight culture, and place the flask in the 37°C incubator with shaking (200 rpm).

   The addition of 0.5 µM EDTA improves K. pneumoniae pelleting.

6. Measure the optical density at 600 nm (OD_{600}) by spectrophotometry starting at 2 hr and then at intervals of 0.5 to 1 hr until the culture reaches an OD_{600} of 0.5-0.6. Bacterial growth can be measured by aseptically removing 1 ml of culture, pipetting it into a cuvette, and reading the absorbance at 600 nm.

   Note that the OD_{600} may quickly exceed 0.6 once it reaches ∼0.4, so measuring the OD_{600} of the culture every 20 min at that point is necessary.

   K. pneumoniae strain KPPR1 typically reaches an OD_{600} of 0.5-0.6 within 2-3 hr at 37°C.

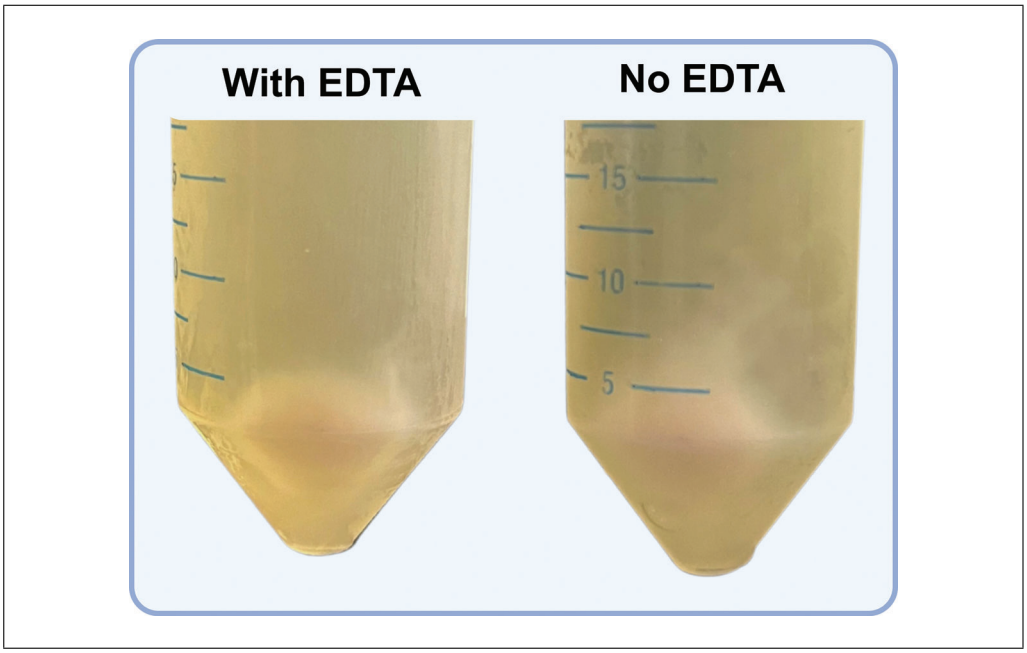

Figure 1 An image capturing the pelleting process of KPPR1 bacterial cells, illustrating the impact of the addition of 0.5 μM EDTA (left) compared to the absence of EDTA (right). The KPPR1 strain, which is hypermucoid, typically forms a loosely packed pellet. However, the inclusion of EDTA enhances the pelleting efficiency. The left image demonstrates the tighter pellet achieved with EDTA present, showcasing its ability to improve the pelleting process during the preparation of competent cells.

Preparation of electrocompetent cells

7. Remove the flask from the incubator and place it on ice for a minimum of 30 min.

   Prechill a sterile 50-ml conical tube on ice alongside the culture to maintain cold conditions throughout the process and ensure efficient competent cells.

8. Transfer the chilled culture to the sterile, cold 50-ml conical tube and centrifuge 15 min at 14,000 × *g*, 4°C.

9. Decant the supernatant and resuspend the cells in a total volume of 20 ml of ice-cold sterile 1 mM HEPES pH 7.4.

   Be cautious when decanting, as K. pneumoniae strains tend to form loose pellets (Fig. 1). Err on keeping some of the supernatant to avoid losing the pellet.

   Allow a 10- to 15-min incubation on ice at each step after decanting and adding solutions to enhance the efficiency of the competent cells.

10. Centrifuge the 50-ml conical tube(s) for 15 min at 14,000 × *g*, 4°C. Decant the supernatant and resuspend the cells with 20 ml of ice-cold sterile ultrapure water.

11. Centrifuge the tube(s) for 15 min at 14,000 × *g*, 4°C. Decant the supernatant and resuspend the cells with 4 ml of ice-cold sterile 10% glycerol.

12. Centrifuge the for 15 min at 14,000 × *g*, 4°C. Decant the liquid carefully and resuspend cells with ice-cold sterile 10% glycerol to a final volume of 0.5 ml, including any residual glycerol from the previous spin.

13. At this point, the competent cells may be electroporated by proceeding to Basic Protocol 2, step 2, or may be saved for later use by following steps 14 and 15 below.

14. Label and then aliquot 50- to 300-μl cell suspensions into sterile 1.7-ml microcentrifuge tubes kept on ice.

Work swiftly during aliquoting to keep the cells cold.

15. Flash-freeze the microcentrifuge tubes using liquid nitrogen and store the cells at −80°C.

    Alternatively, a dry ice/ethanol bath can be used for flash freezing. Add 95%-100% ethanol to a Styrofoam box, add dry ice until the temperature is below −70°C, and then add the tubes for flash freezing. Immediately store them in the −80°C freezer. Electrocompetent cells can remain stored at −80°C for ∼3-6 months for high efficiency transformations, but we have had electrocompetent cells work after 3 years.

PREPARING ELECTROCOMPETENT *K. pneumoniae* CELLS FOR RECOMBINEERING

ALTERNATE PROTOCOL 1

This protocol has been optimized from previously published methodologies and details the preparation of electrocompetent cells from KPPR1 containing pKD46-sp^r for efficient electroporation followed by λ Red recombineering (Bachman et al., 2015; Datsenko & Wanner, 2000). Unlike Basic Protocol 1, the preparation of KPPR1 containing pKD46-sp^r involves a temperature-sensitive origin of replication and an arabinose-inducible promoter, essential for generating isogenic mutants using the λ Red recombination system. The procedure includes overnight culture growth, cell centrifugation, resuspension in HEPES buffer and glycerol, and final storage at −80°C. Following this method ensures that the electroporation efficiency is optimized for subsequent genetic manipulation studies.

Additional Materials (also see Basic Protocol 1)

K. pneumoniae ATCC 43816 (KPPR1) containing pKD46-sp^r
Low-salt LB agar plates (see recipe) with 50 µg/ml spectinomycin (see recipe)
50 mg/ml spectinomycin (see recipe)
50 mM L-arabinose, sterile filtered (see recipe)

Shaking incubator (e.g., USA Scientific, New Brunswick Innova 44), 30°C
250-ml Erlenmeyer flasks (Fisher Scientific, cat. no. 10-040F)
250-ml Nalgene bottles, sterile (Fisher Scientific, cat. no. 02-923-7), prechilled on ice

Culture and growth conditions

1. Streak *K. pneumoniae* strain KPPR1 containing pKD46-sp^r from the −80°C glycerol stock onto a low-salt LB-spectinomycin (50 µg/ml) agar plate and incubate overnight at 30°C.

   The pKD46-sp^r plasmid has a temperature-sensitive origin of replication and must be grown at 30°C. If cells are grown at >30°C, the plasmid can be lost from the cells.

2. Inoculate a single colony of KPPR1 containing pKD46-sp^r into two culture tubes each containing 3 ml of low-salt LB broth, 0.5 µM EDTA, and 50 µg/ml spectinomycin. Incubate the culture overnight at 30°C with continuous shaking (200 rpm).

   K. pneumoniae forms loose pellets and EDTA aids in pelleting to minimize loss when decanting (Fig. 1).

3. Prepare two sterile 250-ml flasks with 125 ml low-salt LB broth for each flask and incubate at 37°C overnight.

4. Aseptically remove 1 ml from both 250-ml flasks containing low-salt LB broth and pipet it into a cuvette to serve as a blank for step 6.

5. Inoculate the sterile 250-ml flasks containing 125 ml of low-salt LB broth with 0.5 µM EDTA, spectinomycin (50 µg/ml), and 50 mM sterile-filtered L-arabinose.

Swirl to mix, and then add 1 ml of the overnight culture and place the flask in the 30°C incubator with shaking (200 rpm).

6. Measure the optical density at 600 nm (OD_{600}) starting 3 hr and then at intervals of 0.5-1 hr until the culture reaches an OD_{600} of 0.5-0.6. Bacterial growth can be measured by aseptically removing 1 ml of culture, pipetting it into a cuvette, and reading the absorbance at 600 nm.

   For the KPPR1 containing pKD46-sp^r to reach 0.5-0.6 typically takes ∼4 hr.

Preparing and storing competent cells

7. Place the culture on ice for a minimum of 30 min.
8. Transfer the chilled culture to a prechilled sterile 250-ml Nalgene bottle and centrifuge 15 min at 14,000 × *g*, 4°C.
9. Decant the supernatant and resuspend cells in a total volume of 100 ml of ice-cold sterile 1 mM HEPES, pH 7.4.

   Be cautious when decanting, as K. pneumoniae strains tend to form loose pellets.

   Allowing a 10- to 15-min incubation on ice at each step after decanting and resuspending the pellets will enhance the efficiency of the competent cells for electroporation.

10. Centrifuge the 50-ml conical tube(s) for 15 min at 14,000 × *g*, 4°C. Decant the supernatant and resuspend the cells in a total volume of 100 ml of ice-cold sterile ultra-pure water.
11. Centrifuge the tube(s) for 15 min at 14,000 × *g*, 4°C. Decant the supernatant and resuspend the cells in a total volume of 20 ml of ice-cold sterile 10% glycerol.
12. Centrifuge the tube(s) for 15 min at 14,000 × *g*, 4°C. Decant the liquid carefully and resuspend cells with ice-cold sterile 10% glycerol to a final volume of 2 ml, including any residual glycerol from the previous spin.
13. Aliquot 50-300 µl of cell suspensions into sterile 1.7-ml microcentrifuge tubes; keep on ice for immediate use or proceed to the next step for storage.

    Work swiftly during aliquoting to keep the cells cold.

14. Flash-freeze the microcentrifuge tubes using liquid nitrogen or dry ice and ethanol and store the cells at −80°C for 3-6 months.

BASIC PROTOCOL 2

TRANSFORMING *K. pneumoniae* USING ELECTROPORATION

This protocol describes a method for introducing DNA into competent *K. pneumoniae* cells using electroporation. Electroporation is a widely employed technique that enables the efficient and precise delivery of foreign DNA into bacterial cells. By utilizing this method, researchers can genetically manipulate *K. pneumoniae* for various applications, such as gene expression studies and the generation of recombinant strains. Briefly, plasmid DNA is added to competent *K. pneumoniae* cells, which are pulsed with an electroporator and allowed to recover at 30°C for 1-2 hr. The recovered cells are then plated on a low-salt LB-antibiotic plate and incubated overnight for the identification of colonies that have successfully taken up the plasmid. Throughout the procedure, stringent aseptic techniques are followed to prevent contamination. By following this protocol, researchers can achieve efficient and reliable transformation of *K. pneumoniae* using electroporation, facilitating various genetic studies and applications.

Materials

Electrocompetent *K. pneumoniae* cells (KPPR1 or other strain of interest; Basic Protocol 1 or Alternate Protocol 1)
10% (v/v) glycerol, sterile
1-5 μl (10 pg-100 ng) plasmid DNA (e.g., pACYC184)
Low-salt LB broth (see recipe)
Low-salt LB agar (see recipe) supplemented with appropriate antibiotics
25-mm-diameter mixed cellulose esters (MCE) membrane (Millipore Sigma, cat. no. VSWP02500)

1.7-ml microcentrifuge tubes, sterile (Genesee Scientific, cat. no. 24-282C)
0.1-cm electroporation cuvette (USA Scientific, cat. no. 9104-1050)
Xcell gene pulser (Bio-Rad, cat. no. 1652662)
Shaking incubator (e.g., New Brunswick Innova 44), 30°C
100 × 15-mm petri dishes (USA Scientific, cat. no. 8609-0010)

Dialyzing plasmid DNA sample and electroporating

1. Thaw three vials each containing 50 μl of electrocompetent cells from the −80°C freezer on ice for 5-10 min.

   IMPORTANT: *From this step on, carry out all steps on ice to ensure efficient transformation. Alternatively, freshly made electrocompetent cells may be used immediately and usually have a higher transformation efficiency.*

2. Label 1.7-ml microcentrifuge tubes: a negative control tube (NC), a positive control tube (PC), and the tube receiving the plasmid DNA (PD).

3. Add 100-200 ng of purified plasmid DNA suspended in sterile water to the 50 μl of competent *K. pneumoniae* cells, usually 5 μl. For the negative control (no DNA), add an equal volume of 10% glycerol and for the positive control, add 1 μl of a plasmid that has been established to transform efficiently (e.g., pCR2.1, pBAD18, or pACYC184) to one vial of competent cells.

   When adding the DNA to the electrocompetent cells, mix by gently flicking the tube. The DNA volume added should not exceed 10% of the volume of cells.

4. Incubate the samples on ice for 30 min and place the 0.1-cm electroporation cuvettes on ice.

   Each transformation requires one electroporation cuvette, so at least three cuvettes are typically needed (a positive control, a negative control, and the sample).

   The best practice is to incubate the cells on ice for 30 min, but we've had success with as little as a 5-min incubation.

5. Transfer the competent cells/DNA mixture to the 0.1-cm electroporation cuvette and electroporate at 1.8 kV, 400 Ω, 25 μF.

6. Immediately add 500 μl low-salt LB broth to the 0.1-cm electroporation cuvette and then transfer the entire sample to the corresponding 1.7-ml microcentrifuge tube labeled in step 2.

Recovering and plating of transformed cells

7. Place the tubes in the 30°C shaking incubator for 1-2 hr.

8. Plate 100 μl and 10 μl of the sample onto low-salt LB agar plates supplemented with appropriate antibiotics for selection purposes.

   Leave the remaining recovered cells on the benchtop overnight, and if transformation efficiency is low, plate the remaining cells again the next morning per step 9.

9. Plate 200 µl of each of the positive and negative control onto two low-salt LB-2% agar plates supplemented with appropriate antibiotics.

   To calculate the transformation efficiency, divide the number of colonies on the transformed plate by the amount of plasmid used (in µg) and then multiply by the final volume used to recover the competent cells divided by the volume of cells plated. For example, 35 colonies were counted on the transformed plate and 0.01 µg of plasmid was used for the transformation. The resuspended volume was 500 µl and 100 µl was plated, and therefore the transformation efficiency is 1.75 × 10^4 transformants/µg DNA.

10. Incubate the plates overnight at 30°C.

11. Remove the plates from the incubator. Cells transformed with the plasmid DNA will have colony-forming units (CFUs) on the LB-antibiotic plates, whereas the negative controls should not have any CFUs.

BASIC PROTOCOL 3

CONSTRUCTING ISOGENIC MUTANTS IN *K. pneumoniae* USING THE λ RED RECOMBINASE SYSTEM

This protocol has been adapted and optimized from previously published methodologies and describes the step-by-step procedure for generating isogenic mutants in *K. pneumoniae* using the λ Red recombinase system (Bachman et al., 2015; Datsenko & Wanner, 2000). The pKD46-sp^r plasmid encodes the λ Red system, which enables accurate and efficient homologous recombination between the knockout antibiotic cassette and a specific gene site in the chromosome. Briefly, this protocol outlines PCR amplification of the kanamycin cassette from the pKD4 plasmid, purification of PCR products, transformation via electroporation, and selection of colonies with the generated knockout with the kanamycin cassette. The expected outcome is the generation of desired mutants with an antibiotic-resistance cassette.

Materials

Purified pKD46-sp^r plasmid (1 pg-10 ng; Addgene, cat. no. 62656)
10 mM dNTPs (Promega, cat. no. U1511)
OneTaq Quick-Load DNA Polymerase and appropriate buffer (New England Biolabs, cat. no. M0509L)
Nuclease-free water, room temperature and 42°C
Agarose type I, low EEO (Millipore Sigma, cat. no. A6013)
1× Tris/acetate/EDTA (TAE) buffer (see recipe)
Biotium GelRed nucleic acid gel stain, 10,000× (Fisher Scientific, cat. no. NC9594719)
Quick-Load Purple 1 kb Plus DNA Ladder (New England Biolabs, cat. no. N0550L)
GenCatch PCR cleanup kit (Epoch Life Science, cat. no. 2360250)
DpnI (New England Biolabs, cat. no. R0176L)
Low-salt LB broth (see recipe)
25 mg/ml kanamycin (see recipe)
Low-salt LB agar plates (see recipe) with 25 µg/ml kanamycin
3 M sodium acetate, pH 5.2 (see recipe)
100% ethanol
70% (v/v) ethanol, ice cold
50% (v/v) glycerol, sterile

Sterile PCR tubes
Thermal cycler (e.g., Applied Biosystems Pro-flex 3 × 32-well PCR System)
Horizontal gel casting system including, caster, combs, gel box, and power supply (Bio-Rad, cat. no. 1640300)

Table 1 Sequences of the Oligonucleotide Primers Specific for pKD4 P1 and P2 Sites and the *rmpD* Gene[a,b]

Oligonucleotide primers	Sequence
Forward primer (**P1**)	5′-AAGAAATGGTTGATGAAAGATGTCTCATGCTAGGTATTTAGAAAAAAAGGGGAGGA GGGG **GTGTAGGCTGGAGCTGCTTC**-3′
Reverse primer (**P2**)	5′-ATGCAATATGCATACTTATCTGCCAACTAAAATTGTCGTGAGTTATATACCGCGCTGATA **ATGGGAATTAGCCATGGTCC**-3′

[a] P1 and P2 sites of pKD4 are underlined and boldface.
[b] Additional primer examples for making gene deletions are detailed in Table S1 of Mike et al. (2021).

Microwave
UV transilluminator (e.g., D.A.I. Scientific Equipment, cat. no. E3000)
1.7-ml microcentrifuge tubes, sterile (Genesee Scientific, cat. no. 24-282C)
Fume hood
1.8-ml cryovials (Genesee Scientific, cat. no. 24-203P)

PCR amplification of the kanamycin cassette

1. Design oligonucleotide primers specific for the P1 and P2 sites on pKD4 for the amplification of the kanamycin cassette. Primer examples can be found in Table 1 for the example gene, *rmpD* (Khadka et al., 2023).

   Add 60 bases matching the sequence preceding or at the start of the gene of interest for the 5′ end of P1 sequence (5′-GTGTAGGCTGGAGCTGCTTC). Also, add 60 bases matching the reverse complement sequence preceding or at the end of the gene of interest for the 5′ end of the P2 sequence (5′-ATGGGAATTAGCCATGGTCC).

   Avoid using the pKD3 plasmid due to poor selection when using chloramphenicol for K. pneumoniae knockouts. Additionally, you can use the pACYC184 plasmid to amplify a tetracycline cassette. Note, this does not contain FRT sites required for Basic Protocol 4.

2. Prepare working stocks of primers at 50 μM in nuclease-free water.

3. Set up six 50-μl PCR reactions (300 μl total) containing the following:

   2 μl purified pKD4 (1 pg-10 ng)
   0.2 μl of each 50 μM primer
   1 μl of 10 mM dNTPs
   10 μl of 5× OneTaq Standard Reaction Buffer
   0.25 μl Taq polymerase
   36.6 μl nuclease-free water.

   Setting up six reactions is important to ensure a sufficient yield of PCR product for electroporation.

4. Set up the following conditions on the thermocycler:

Initial step:	5 min	94°C (denaturation)
30 cycles:	1 min	94°C (extension)
	1 min	51°C (annealing)
	30 s	68°C (extension)
Final step:	5 min	68°C (extension).

   The kanamycin cassette in the pKD4 plasmid is 1496 base pairs.

5. Prepare a 0.8% (w/v) agarose gel by dissolving 0.24 g agarose in 30 ml of 1× TAE buffer. Microwave on high until fully dissolved.

   Typical agarose concentrations range from 0.8% to 1.2%, with higher percentages enhancing the resolution of lower-molecular-weight bands and lower percentages providing better resolution for higher-molecular-weight bands.

6. Add 1.2 μl GelRed into the slightly cooled 1% agarose gel. Pour the gel into a gel apparatus, add the gel combs, and allow it to fully polymerize.

   The gel will be fully polymerized in ∼15 min.

7. Pipet 5 μl of each PCR reaction and the DNA ladder into the polymerized 1% agarose gel. Run at 130 V for ∼25 min.

   Adjust the voltage to run the gel more slowly, if desired. Adjust the time accordingly.

8. Visualize the gel on a DNA transilluminator to confirm that the PCR product is amplified at 1496 base pairs.

Preparation of recombinase substrate

9. Pool together the six PCR reactions containing identical PCR products (∼300 μl/gene) into a sterile 1.7-ml microcentrifuge tube.

10. Purify the pooled PCR products using a GenCatch PCR Cleanup Kit. Elute with 35 μl prewarmed nuclease-free water at the final step.

    If >100 μl of DNA needs to be cleaned up, adjust the amount of first buffer in the kit, PX Buffer, to add 5 μl more PX Buffer for each 1 μl of extra DNA solution. For example, 300 μl of DNA solution will require 1.5 ml of PX Buffer.

    Warming the nuclease-free water at 42°C aids in sample elution.

11. Digest the purified PCR products with DpnI by adding 3.5 μl of 10× CutSmart reaction buffer (supplied with the enzyme) and 2 μl DpnI to the pooled purified PCR samples and placing them into the thermal cycler for 2-8 hr at 37°C, and then heat-inactivate at 80°C for 20 min.

    DpnI treatment degrades template DNA (which is methylated) but does not digest the PCR product.

    If the digestion is performed overnight, hold at 4°C after heat inactivation.

12. Purify digestions using a GenCatch PCR Cleanup Kit as described above; however, elute with 15 μl sterile nuclease-free water at the final step.

13. Store the purified product at −20°C.

Electroporation and selection for recombinant clones

14. Thaw 50 μl of competent KPPR1 pKD46-sp^r competent on ice.

15. Add 6 μl (∼1/2 eluate) of the purified and digested PCR product (from step 13) to the thawed competent cells. Gently mix the mixture by flicking the tube.

16. Perform electroporation following the steps outlined in Basic Protocol 2, steps 4-9.

17. Allow cells to recover in 500 μl of low-salt LB broth at 30°C with shaking for 3 hr in sterile 1.7-ml microcentrifuge tubes.

18. Plate 200 μl of the cells onto low-salt LB agar plates supplemented with 25 μg/ml kanamycin. Incubate the plates at 37°C overnight. Patch any potential mutant colonies on LB agar plates with 25 μg/ml kanamycin and then confirm the knockout via colony PCR (Support Protocol 1).

    After step 18 is complete, leave the excess recovered cells on the benchtop overnight to continue the recovery process. If the transformation plates from step 18 fail to yield colonies, plate the overnight samples on the following day. It has been observed that allowing cells to recover on the bench overnight can increase colony yield.

The next day, if colonies appear, the transformation was successful, and then steps 19-23 are not necessary.

Recovery and concentration of cells

19. Examine the agar plates the next day. If no colonies appear on the agar plates, recover the remaining cells by centrifugation and remove the supernatant. Resuspend the pelleted cells with 100 μl low-salt LB and plate as described in step 18.

20. Examine the new agar plates the next day. If there are still no colonies visible, concentrate the remaining purified and digested PCR product as follows: bring reactions to 125 μl with ultrapure water, add 12.5 μl of 3 M sodium acetate (pH 5.2), mix well, and add 375 μl ice-cold 100% ethanol. Incubate at −80°C for 30 min and centrifuge 10 min at 16,000 × *g*, 4°C.

21. Carefully remove all liquid with a 150-μl pipet. Add 500 μl of ice-cold 70% ethanol to the pellet. Briefly vortex the tube to ensure proper mixing and then centrifuge 10 min at 16,000 × *g*, 4°C.

22. Carefully remove all liquid with a small pipet as before. Place the tube with the cap open in a fume hood for 10-20 min, allowing the ethanol to evaporate (do not over-dry).

23. Add 50 μl of thawed pKD46-sp^r cells to the dried pellet and then incubate on ice and electroporate as described in step 16.

Storage of isogenic mutants

24. Select a single colony of the transformed mutant strain, inoculate into one culture tube containing 3 ml of low-salt LB broth with 25 μg/ml kanamycin, and incubate the culture overnight at 37°C with shaking (200 rpm).

    Selecting a single colony to save a strain ensures genetic purity and allows researchers to maintain a well- defined and reliable genetic entity for subsequent experiments.

25. Pipet 0.5-0.9 ml of the overnight culture into a 1.8-ml cryovial. Add an equal volume of sterile 50% glycerol to the culture in the cryovial and mix well. Store the cryovial containing the culture and glycerol mixture in the −80°C freezer for long-term storage.

CONFIRMING A KNOCKOUT VIA COLONY PCR

SUPPORT PROTOCOL 1

This protocol outlines a procedure for efficient screening and selection of knockout mutants generated from Basic Protocol 3 using colony PCR. By lysing of bacterial cells, PCR amplification, and visualization of PCR products, this approach enables high-throughput and reliable screening of bacterial colonies, facilitating the identification of target genetic manipulations. This protocol provides a detailed step-by-step guide to ensure successful screening and selection of knockout mutants.

Materials

K. pneumoniae strain KPPR1 with an isogenic mutant (Basic Protocol 3)
Wild-type *K. pneumoniae* KPPR1
Ultrapure water
Low-salt LB agar (see recipe) with 25 μg/ml kanamycin (see recipe)
Forward and reverse primers specific for DNA sequence of interest
10 mM dNTPs (Promega, cat. no. U1511)
OneTaq Quick-Load DNA Polymerase (New England Biolabs, cat. no. M0509L)
Nuclease-free water

Table 2 Oligonucleotide Sequences Spanning the Entire *aceE* Gene and Adjacent Genomic Content[a,b]

Oligonucleotide primers	Sequence
Forward primer (*aceE::*tcR-check*)*	5′-GCAACTAAACGCAGAACCTGTC-3′
Reverse primer (*aceE::*tcR-check*)*	5′-CTTCTACTTTGTCGCCAACTTTGAC-3′

[a] Amplification of a ~1.5-kb fragment indicates a successful generation of an isogenic mutant, whereas amplification of a ~2.8-kb fragment indicates that wild-type *aceE* is present.
[b] Additional primer examples for checking gene deletions are detailed in Table S1 of Mike et al. (2021).

PCR tubes, sterile
Toothpicks, sterile
30°C static incubator (e.g., Fisher Scientific, Heratherm™)
Microwave
−20°C and −80°C freezers
PCR tubes, sterile
Thermal cycler (e.g., Applied Biosystems Pro-flex 3 × 32-well PCR System)
Horizontal gel casting system including, caster, combs, gel box, and power supply (Bio-Rad, cat. no. 1640300)
Accuris UV transilluminator (e.g., D.A.I. Scientific Equipment, cat. no. E3000)
1.8-ml cryovials (Genesee Scientific, cat. no. 24-203P)

Cell lysis

1. Use a sterile toothpick to inoculate four sterile PCR tubes containing 100 µl ultrapure water each with a single colony expected to be an isogenic mutant. Inoculate one sterile PCR tube containing 100 µl ultrapure water with a single colony of wild-type KPPR1 as a control.

   Screening more colonies via colony PCR provides increased chances of identifying knockouts to send for sequencing for confirmation. If screening many colonies (>20), a sterile 96-well plate can be used instead of PCR tubes.

   Wild-type KPPR1 serves as a control to allow the easy identification of colonies that carry the targeted gene disruption.

2. Streak the same colonies onto a low-salt LB-kanamycin (25 µg/ml) agar plate and incubate the plate at 30°C overnight.

3. Boil the inoculated PCR tubes for 1 min on high in a microwave.

4. Incubate the PCR tubes at −20°C until fully frozen.

   For a faster freeze, the tubes can be incubated at −80°C.

5. Thaw the samples at room temperature and proceed to PCR.

   Boiling the samples in the microwave followed by a freeze-thaw cycle is a quick method for lysing cells to obtain template DNA for PCR.

PCR amplification of the target sequence

6. Design primers that anneal adjacent to the targeted DNA sequences.

   In Figure 2, the aceE gene was replaced with a tetracycline cassette following Basic Protocol 3 using pACYC184 instead of pKD4 for template. See Table 2 for example primers.

7. Prepare working stocks of primers at 10 µM.

   Primers are designed to target the entire gene sequence; therefore, they are specific to the sequences that are immediately upstream and downstream of the gene. These primers will allow the identification of mutants that have been created as there will be a distinct number of base pairs in a mutant compared to wild type, which is visualized on a DNA gel.

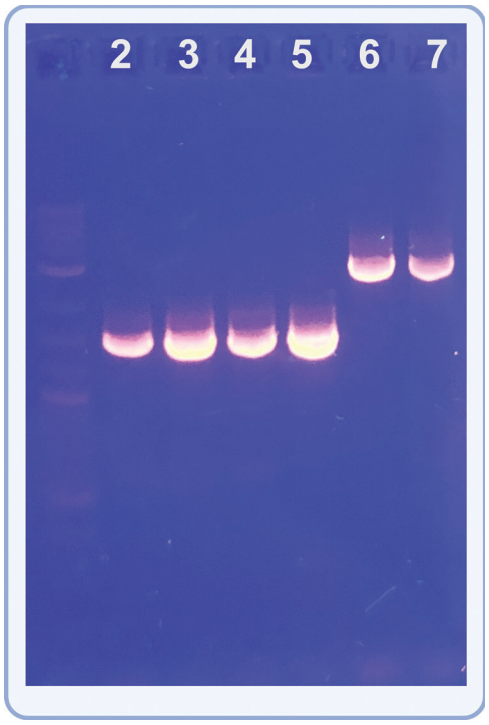

Figure 2 Gel electrophoresis visualization of colony PCR results to validate the presence and modifications of a mutant. Primers flanking each side of the *aceE* gene-deletion site were used, yielding products of ~1.5 kb for successful mutants and ~2.8 kb for wild-type strains. From left to right: lanes 2-5 display successful *aceE* gene replacement with the antibiotic cassette, lane 6 represents an unsuccessful attempt, and lane 7 serves as the wild-type KPPR1 control.

8. Prepare five separate PCR reactions by combining 1 µl of each lysed DNA template, nuclease-free water, a PCR buffer, forward and reverse primers, dNTP mix, and DNA polymerase enzyme in a total volume of 25 µl, according to the manufacturer's directions.
9. Set up the thermal cycler program according to the specific PCR conditions required for the amplification of the targeted DNA sequence of interest.
10. Visualize the PCR products following the steps outlined in Basic Protocol 3, steps 5-8 (Fig. 2).
11. Select colonies from the low-salt LB-kanamycin (25 µg/ml) agar plate that have PCR amplified at the correct size for the isogenic mutant and save the strain following steps 24 and 25 in Basic Protocol 3.

    If the PCR product of the mutant has a similar size to the wild type, a restriction digest can be used to differentiate the two, as the kanamycin cassette that replaces the gene of interest has a single EagI cut site.

VERIFYING ABSENCE OF SECONDARY MUTATIONS

SUPPORT PROTOCOL 2

This protocol describes a method to analyze mutations using a web-based tool, BV-BRC. As the capsule locus is a hot spot for mutations, it is important to validate that a mutant has no secondary mutations. Briefly, a whole genome sequence of the strain of interest is submitted to BV-BRC (Olson et al., 2023). The tool will align the sequence against a reference genome selected by the user and generate a result consisting of all locus variants, including the capsule locus. This tool provides a useful and simple bioinformatic pipeline for variant calling.

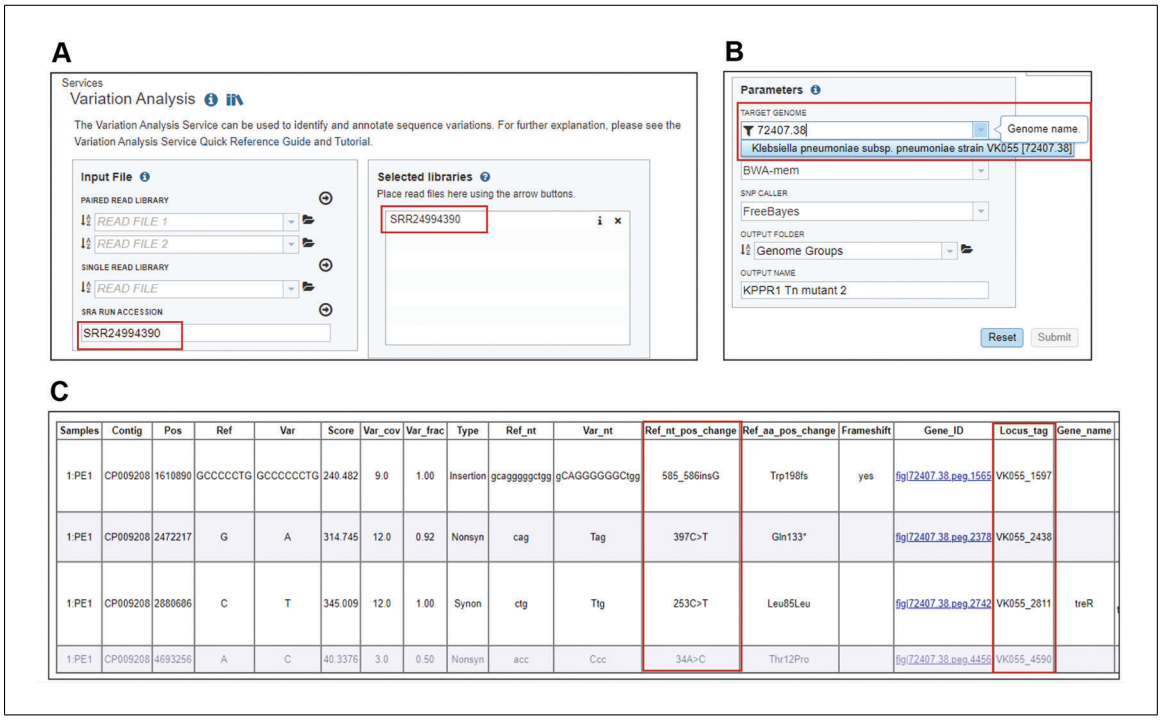

Figure 3 Representative screen captures of KPPR1 variation analysis using BV-BRC. (**A**) Whole-genome sequence to be analyzed is input in the 'Input File' box. SRR24994390 is used for illustration. (**B**) Analysis is performed against a reference sequence specified in 'TARGET GENOME'. SRR24994390 is compared against the reference genome of '*Klebsiella pneumoniae* subsp. *pneumoniae* VK055 [72407.38]' in the example shown. (**C**) A list of identified variants is generated at the end of the analysis. Variations in the loci VK055_1597, VK055_2438, VK055_2811, and VK055_4590 were identified in SRR24994390.

Necessary Resources

Short-read or assembled whole-genome sequence or SRR accession of SRA-deposited sequence of *K. pneumoniae*

Bacterial and Viral Bioinformatics Resource Centre, version 3.30.19 (BV-BRC; *https://www.bv-brc.org/*)

Desktop or laptop computer (no tablets) with a minimum screen resolution 1376 × 768 and running the Chrome, Safari, or Firefox browser

Variation analysis using BV-BRC

1. Sign up and log in to BV-BRC.
2. Open 'Variation Analysis (B)' from the 'TOOLS & SERVICES' tab at the top of the homepage. A new page to submit the sequence will load (Fig. 3A).
3. Input the whole-genome sequence file by uploading the sequence file or enter an SRA Run accession number in the 'Input File' dialog box (Fig. 3A). For example, enter SRR24994390 to analyze variations in a KPPR1 transposon mutant submitted to the SRA database (Khadka et al., 2023). Once entered, click on the right-pointing arrow, beside the text box, to move the sequence to 'Selected libraries' box.

   If the input file is a paired read short-read sequence or single read sequence, upload the file beforehand to a folder within the workspace and simply locate and select the uploaded file in this step. Alternatively, the sequence can be both uploaded and selected in this step. Click on the upload button located at the upper right corner of 'Choose or Upload a Workspace Object' dialog box. Depending on the file size, uploading can take several minutes. The upload progress can be tracked from the bottom right corner of the page.

4. Select, in the parameters box, the reference strain under 'Target Genome' (Fig. 3B). Similarly, in the 'Output Folder' dropdown box, select the location where the output

is to be saved after analysis. Next, enter the name of the output file. For our example, enter 72407.38 in the Target Genome textbox and select '*Klebsiella pneumoniae* subsp. *pneumoniae* strain VK055 [72407.38]' from the list, if the parental strain is an ATCC 43816 derivative.

5. Click 'Submit' to start the analysis. Depending on the number of variants, server availability, and genome size, the whole analysis can take from a few minutes to several days to complete.

   The analysis progress can be tracked from the bottom right corner. Alternatively, click on the 'Jobs' box at the bottom right corner to open a new page with a list of all jobs submitted for analysis.

6. Once the analysis is complete, double-click on the job name to open the completed analysis files.

7. Locate from the list 'all.var.html' and open it by double-clicking. The new page will contain a list of all the variations found by BV-BRC Variation Analysis (Fig. 3C).

8. Confirm whether any non-synonymous secondary mutations are present in the list, especially in the capsule biosynthesis locus.

9. Download the analysis by clicking on the 'DWNLD' button on the right of the table.

GENERATING UNMARKED KNOCKOUT MUTANTS IN *K. pneumoniae* USING THE PFLP PLASMID

BASIC PROTOCOL 4

This protocol has been optimized from previously published methodologies and presents a comprehensive method for generating unmarked knockout mutants in *K. pneumoniae* using the pFLP-hyg plasmid (Chen et al., 2021; Huang et al., 2014). The pFLP-hyg plasmid harbors FLP recombinase, which facilitates the precise excision of the kanamycin selection marker flanked by FRT sites. However, for the FLP recombinase to work, the antibiotic cassette must contain the FRT sites. By employing the pFLP system, this protocol enables the removal of the antibiotic cassette introduced in Basic Protocol 3. Note that the induction of the FLP recombinase and then subsequent curing of the pFLP plasmid are done at 42°C, whereas checking for hygromycin-sensitive patches is done at 30°C. Furthermore, safety considerations are crucial throughout the procedure. It is important to maintain sterility when handling bacterial cultures and follow dedicated safety protocols for high-voltage electroporation. The protocol includes the preparation of electrocompetent cells, transformation via electroporation, induction of FLP recombinase, multi-day screening for kanamycin and hygromycin sensitivity, and confirmation of clean knockout in the mutants via colony PCR and sequencing. This approach offers an efficient and reliable method for generating unmarked knockout mutants, serving as a valuable tool for various genetic studies.

Materials

K. pneumoniae strain KPPR1 containing a gene replaced with an FRT-flanked antibiotic (e.g., kanamycin) cassette from Basic Protocol 3
Low-salt LB agar plates (see recipe) with 25 μg/ml kanamycin
Low-salt LB broth (see recipe)
0.5 mM EDTA, pH 8 (see recipe)
25 mg/ml kanamycin (see recipe)
E. coli DH5α containing pFLP-hyg (Addgene, cat. no. 87831)
Low-salt agar plates
Low-salt LB agar plates (see recipe) with 100 μg/ml hygromycin (see recipe)
Nuclease-free water
Confirmation primers designed for the affected chromosomal region

50% (v/v) glycerol, sterile

Static incubator (e.g., Fisher Scientific, Heratherm), 30°C
17 × 100-mm aeration culture tubes (Genesee Scientific, cat. no. 21-131)
Shaking incubator (e.g., New Brunswick Innova 44), 30°C
250-ml Erlenmeyer flask (Fisher Scientific, cat. no. 10-040F)
Static incubator (e.g., Fisher Scientific, Heratherm), 42°C
Static incubator (e.g., Fisher Scientific, Heratherm), 37°C
PCR tubes
Microwave
−20 and −80°C freezers
1.8-ml cryovials (Genesee Scientific, cat. no. 24-203P)

Preparation of electrocompetent cells and transformation with electroporation

1. Streak *K. pneumoniae* strain KPPR1 containing an FRT-flanked kanamycin cassette from the −80°C glycerol stock onto a low-salt LB-kanamycin (25 µg/ml) agar plate and incubate overnight at 30°C.
2. Inoculate a single colony of *K. pneumoniae* strain KPPR1 containing a kanamycin cassette instead of the gene of interest into two culture tubes containing 3 ml of low-salt LB broth, 0.5 µM EDTA, and 25 µg/ml kanamycin.
3. Incubate the culture overnight at 30°C with continuous shaking (200 rpm).
4. Inoculate 1 ml of the overnight culture into a 250-ml Erlenmeyer flask containing 100 ml low-salt LB and 25 µg/ml kanamycin. Prepare electrocompetent cells following the steps outlined in Basic Protocol 1.
5. Perform electroporation following the steps outlined in Basic Protocol 2 (step 4 onward) using the miniprepped pFLP-hyg plasmid. Plate on low-salt LB-hygromycin (100 µg/ml) agar plates and incubate at 30°C.

Induction of FLP recombinase by heat-shocking

6. Select 12-16 colonies present on the low-salt LB-hygromycin (100 µg/ml) agar plates and streak onto a low-salt LB agar plate (without antibiotics). Incubate the plate at 42°C overnight.

   Streaking on the low-salt LB agar plate removes antibiotic pressure and allows the loss of the antibiotic cassette by inducing FLP recombinase expression at 42°C.

Screen colonies for loss of kanamycin resistance

7. Patch the 12-16 colonies isolates from the overnight plate onto a low-salt LB agar plate and incubate at 42°C. Additionally, patch the colonies onto a low-salt LB-kanamycin (25 µg/ml) agar plate and incubate at 30°C overnight.

   Colonies that do not grow on the low-salt LB-kanamycin agar plate have lost the kanamycin cassette via excision by FLP recombinase. Streaking onto the low-salt LB agar plate preserves colonies that have successfully excised the kanamycin cassette (Fig. 4).

Passage colonies to lose the pFLP-hyg plasmid

8. Patch the colony replicates that are kanamycin sensitive onto a low-salt LB agar plate. Incubate the plate at 42°C overnight and repeatedly pass the colonies on low-salt LB agar plates for 3 consecutive days.
9. On the fourth day, patch the colony replicates onto a low-salt LB agar plate and a low-salt LB-hygromycin (100 µg/ml) agar plate to screen for hygromycin sensitivity. Incubate the low-salt LB agar plate at 42°C overnight and the low-salt LB-hygromycin agar plate at 30°C.

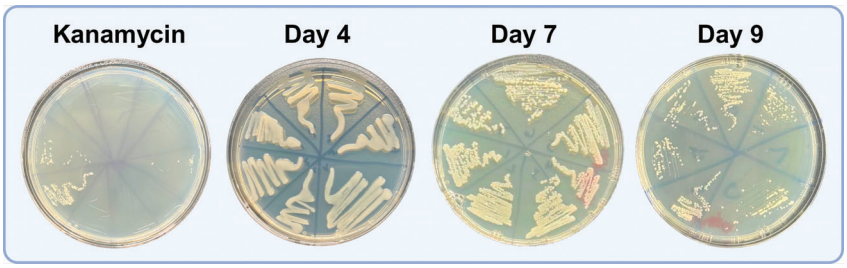

Figure 4 An image capturing kanamycin sensitivity after pFLP induction and then subsequent loss of the pFLP plasmid after multiple passages for an unmarked knockout. From left to right: kanamycin sensitivity in 12 patches, day 4 patches on hygromycin (100 µg/ml) agar plates, day 7 patches on hygromycin (100 µg/ml) agar plates, and day 9 patches on hygromycin (100 µg/ml) agar plates. The kanamycin-resistant patches that have grown indicate that the kanamycin cassette was not removed by FLP recombinase and were not used for subsequent passaging. On day 4 after patching on hygromycin, the patches are dense, but by day 7 the patches begin to show colony density decreasing on visual inspection, and by day 9 (right) hygromycin-sensitivity is seen in one of the seven patches.

Incubating the low-salt LB-hygromycin plate at 30°C will identify colonies that have become hygromycin sensitive again, indicating loss of pFLP-hyg.

10. Check the low-salt LB-hygromycin agar plate for growth. If there is no growth, the pFLP-hyg plasmid has been lost; proceed to step 12. If there is growth, the pFLP-hyg plasmid remains; repeat step 11 until the strain becomes hygromycin sensitive.

11. Patch the colony replicates daily onto a low-salt LB agar plate and a low-salt LB-hygromycin agar plate (100 µg/ml) to monitor for hygromycin sensitivity until the plasmid is lost (Fig. 4).

    Loss of the pFLP-hyg plasmid typically takes 4-10 days. Between days 4 and 7, colonies on the low-salt LB-hygromycin plate will show decreased density, indicating plasmid loss. Additional passaging on low-salt LB agar plates at 42°C for 1-2 days ensures complete plasmid loss.

12. Select four hygromycin- and kanamycin-sensitive colonies, inoculate four culture tubes with 3 ml each of low-salt LB broth, and incubate at 37°C overnight with shaking (200 rpm).

13. Perform colony PCR according to Support Protocol 1, steps 1-9, and save the strains that have lost the kanamycin cassette as described in Support Protocol 1, steps 10 and 11, but do not add kanamycin.

CONSTRUCTING A COMPLEMENTATION VECTOR FOR *K. pneumoniae*

BASIC PROTOCOL 5

This protocol describes the construction and validation of a complementation vector in a *K. pneumoniae* KPPR1 knockout strain to confirm the specific role of a targeted gene in an observed phenotype. Safety hazards include the need for sterile techniques during bacterial culture handling, electroporation, restriction enzyme work, and gel electrophoresis. The protocol involves preparing PCR products, constructing the complementation vector, transforming into *E. coli*, confirming the plasmid, and transforming into *K. pneumoniae* deletion strains. Successfully executing the protocol will yield a complementation vector containing the gene of interest transformed into an isogenic mutant of interest, enabling studies to determine whether the plasmid restores the phenotype of interest.

Materials

K. pneumoniae genomic DNA

Table 3 Oligonucleotide Sequences Used to Generate a *csrD* Complementation Vector[a]

Oligonucleotide primers	Sequence
Forward primer A (amplifies predicted *csrD* promoter and gene)	5′-CATAAGGGAGAGCGTCGACCTGGTGTCCTCCGTCATGC-3′
Reverse primer B (amplifies predicted *csrD* promoter and gene)	5′-TCCATTCAGGTCGAGGTGGCTCAAACCGAGTATCTTTGCG-3′
Forward primer C (amplifies pACYC184 backbone, eliminating *tet*)	5′-GCCACCTCGACCTGAATGG-3′
Reverse primer D (amplifies pACYC184 backbone, eliminating *tet*)	5′-GGTCGACGCTCTCCCTTATG-3′

[a] Additional primer examples for constructing complementation vectors are detailed in Table S1 in Mike et al. (2021).

Purified pACYC184 plasmid (ATCC, cat. no. 38033)
Primers for pACYC184 backbone and gene of interest with promoter Monarch DNA polymerase and buffer
Nuclease-free water, prewarmed to 50°C
DNA gel extraction kit (New England Biolabs, cat. no. T1020L)
NEBuilder HiFi DNA assembly master mix (New England Biolabs, cat. no. E2621X)
10% (v/v) glycerol, sterile
Electrocompetent TOP10 E. coli cells
Low-salt LB agar plates (see recipe)
Low-salt LB agar plates (see recipe) with 20 μg/ml chloramphenicol (see recipe)
GenCatch plasmid DNA mini-prep kit (Epoch Life Science, cat. no. 2160250)
10 mM dNTPs (Promega, cat. no. U1511)
rCutSmart Buffer (New England Biolabs, cat. no. B6004S)
Restriction enzyme(s)
Electrocompetent KPPR1 cells with the mutant gene of interest

1.7-ml microcentrifuge tubes, sterile (Genesee Scientific, cat. no. 24-282C)
PCR tubes, sterile
Thermal cycler (e.g., Applied Biosystems Pro-flex 3 × 32-well PCR System)
Monarch DNA Gel Extraction Kit
Nanodrop spectrophotometer
25-mm-diameter mixed cellulose esters (MCE) membrane (Millipore Sigma, cat. No. VSWP02500)
100 × 15-mm petri dishes (USA Scientific, cat. no. 8609-0010)
0.1-cm electroporation cuvette (USA Scientific, cat. no. 9104-1050)
Xcell Gene pulser (Bio-Rad, cat. no. 1652662)
17 × 100-mm culture tubes (Genesee Scientific, cat. no. 21-131)

PCR amplification

1. Design primers to amplify the pACYC184 backbone and the gene of interest along with its promoter region. Primer examples for the *csrD* gene can be found in Table 3 (Mike et al., 2021).

   The vector backbone should contain the necessary elements for replication, selection, and maintenance in KPPR1. The pACYC184 plasmid contains chloramphenicol and tetracycline resistance genes.

2. Prepare two separate 50-μl PCR reactions for amplifying the vector backbone and two separate 50-μl PCR reactions for amplifying the gene of interest. In each reaction, mix 4 μl of each respective template (10 ng/μl), nuclease-free water, PCR buffer, forward and reverse primers, dNTP mix, and DNA polymerase enzyme.

3. Set up the thermal cycler program according to the specific PCR conditions for amplifying the vector backbone and the gene of interest. Ensure that the annealing temperature, extension time, and number of cycles are appropriate for each reaction.
4. Pool together the two 50-µl PCR reactions, and then visualize the PCR products and verify their sizes following the steps outlined in Basic Protocol 3, steps 5-8.
5. Extract the DNA bands of interest from the agarose gel using the Monarch DNA Gel Extraction Kit, and at the last step in the kit, elute with 12 µl prewarmed nuclease-free water.
6. Measure the concentrations of DNA for both extracted samples using a Nanodrop spectrophotometer or a Qubit system.

Ligation and transformation of the vector and insert

7. Prepare NEBuilder reactions in a sterile PCR tube. Use the NEBiocalculator to determine the 1:2 molar ratios of vector to insert(s). Add 50-100 ng of pACYC184 and the appropriate quantity of inserts to achieve a 1:2 molar ratio of vector to insert. Add 5 µl of 2× NEBuilder Master Mix and bring the final volume to 10 µl with nuclease-free water.
8. Incubate the reaction at 50°C for 60 min using a thermal cycler.
9. Dialyze the NEBuilder reaction using a 0.025-µm MCE filter disk against 10% glycerol (sterile). Perform the dialysis for 2 hr to overnight in a sterile petri dish at room temperature.

   If transforming by heat shock, dialysis is unnecessary. Dialysis removes salts to avoid arcing during electroporation.

10. Recover the dialyzed DNA by adding 4 µl of 10% sterile glycerol to the surface of the dialysis discs. Carefully pipet up and down to ensure DNA recovery.
11. Combine ∼5 µl of dialyzed DNA with 50 µl of electrocompetent TOP10 *E. coli* cells. Appropriate controls include 5 µl of glycerol only as a negative control and 1 µl of 1:10 miniprepped pACYC184 (empty vector) as a positive control.

    A negative control that shows growth indicates there was contamination, and the positive control indicates that the cells are efficient and competent.

12. Incubate the cells on ice for 10 min, and then perform electroporation using 0.1-cm-gap cuvettes with settings of 1.8 kV, 400 Ω, and 25 µF.

    The incubation on ice is shorter for E. coli, at 10 min, than for K. pneumoniae, which shows increased transformation efficiency with 20-30 min of incubation with DNA.

13. Recover the transformed cells by adding 500 µl of low-salt LB medium and then shaking at 30°C for 2-3 hr. Plate 20 µl and 200 µl of the controls, as well as pACYC184 with gene of interest, on separate low-salt LB-chloramphenicol (20 µg/ml) agar plates. Incubate at 30°C overnight.

    Save the excess transformed cells on the benchtop. If transformation yield is low, plate the remainder on low- salt LB-chloramphenicol (20 µg/ml) agar plates.

14. Select four single colonies and patch them onto a low-salt LB-chloramphenicol (20 µg/ml) agar plate. Use the same four colonies to inoculate four culture tubes containing 3 ml of low-salt LB broth and chloramphenicol (20 µg/ml).

    Patching the colonies onto a plate allows strain preservation until the sequences are validated.

15. Perform a miniprep on the pACYC184 complementation vector as described above. Elute in 35 µl of nuclease-free water and store the DNA at −20°C.

Restriction digestion and validation of a complementation vector

16. Prepare a restriction digest by combining 5 µl of the miniprepped plasmid, 1 µl of 10× rCutSmart Buffer, 0.5 µl of the appropriate restriction enzyme(s), and enough nuclease-free water to adjust the volume to 10 µl.

17. Incubate the reaction mixture at the optimal temperature and time recommended for the specific restriction enzyme(s) being used.

    Typical restriction digest conditions are 37°C for 2 hr.

18. Visualize the restriction-digested plasmid by following the steps outlined in Basic Protocol 3, steps 5-8. If the digested plasmid is cut as expected, send off the plasmid for sequencing.

19. Perform the transformation of the pACYC184 complementation vector into electrocompetent KPPR1 cells with the corresponding mutant gene, following the steps outlined in Basic Protocol 2, steps 4-14. Store strains at −80°C as described in Basic Support Protocol 3, steps 24 and 25.

    To maintain the pACYC184 plasmid in K. pneumoniae and E. coli, add chloramphenicol at 80 µg/ml or 20 µg/ml, respectively.

20. Validate the complementation vector with appropriate phenotypic assay(s) to assess effects of the gene of interest on the phenotype(s) of interest.

REAGENTS AND SOLUTIONS

L-Arabinose, 50 mM

Dissolve 0.75 g L-arabinose in 75 ml ultrapure water. Adjust the volume to 100 ml and filter sterilize with a 0.2-µm filter. Store up to 1 year at 4°C.

Chloramphenicol, 20 mg/ml and 80 mg/ml

Dissolve 0.2 g chloramphenicol to make 20 mg/ml stock for use with *E. coli*, or 0.8 g chloramphenicol to make 80 mg/ml stock for use with *K. pneumoniae*, in 10 ml of 100% ethanol and freeze aliquots. Stocks may be kept up to 1 year at −20°C.

Ethylenediaminetetraacetic acid (EDTA), pH 8, 0.50 mM

Add 0.146 g EDTA to 750 ml ultrapure water with stirring. Adjust the pH to 8 with 10 M NaOH. Once dissolved, bring volume up to 1000 ml, and autoclave. Store up to 1 year at room temperature.

EDTA will only dissolve at pH 8.

HEPES, pH 7.4, 0.50 mM

Dissolve 0.238 g of HEPES into 750 ml ultrapure water with stirring. Adjust the pH to 8 with 10 M NaOH; once dissolved, bring volume up to 1000 ml and autoclave. Store up to 1 year at 4°C.

Hygromycin, 100 mg/ml

Dissolve 1 g hygromycin in 10 ml ultrapure water. Filter sterilize with a 0.2-µm filter, aliquot, and freeze. Stock may be kept up to 1 year at −20°C.

Kanamycin, 25 mg/ml

Dissolve 0.25 g kanamycin in 10 ml ultrapure water. Filter sterilize with a 0.2-µm filter, aliquot, and freeze. Stock may be kept up to 1 year at −20°C.

Low-salt LB agar

5 g yeast extract
10 g tryptone
0.5 g sodium chloride
20 g agar

Add all reagents and then ultrapure water to a final volume of 1000 ml in a 20-L flask. Stir on a hot plate until the solids have completely dissolved and then autoclave for 20 min. Allow to cool until the medium is still liquid, but cool enough to be comfortably held (∼55°C-65°C). Add antibiotics, if needed (e.g., chloramphenicol, hygromycin, kanamycin, or spectinomycin; see recipes), once the medium has cooled and mix well. Pour ∼25-ml portions into sterile petri dishes and allow to solidify. Leave plates sit for 1-2 days at room temperature until sufficiently dry. Store in a sealed bag up to 3 months at 4°C.

Antibiotics can be sensitive to light.

Low-salt LB broth

5 g yeast extract
10 g tryptone
0.5 g sodium chloride

Add all reagents and ultrapure water to a final volume of 1000 ml in a 4-L beaker. Stir until the solids have completely dissolved. Distribute into a clean bottle and autoclave for 20 min. Store at room temperature for up to 3 months.

Spectinomycin, 50 mg/ml

Dissolve 0.5 g spectinomycin in 10 ml ultrapure. Filter sterilize using 0.2-μm filters and freeze aliquots. Stock may be kept up to 1 year at −20°C.

Sodium acetate, pH 5.2, 3 M

Dissolve 24.6 g of sodium acetate with 70 ml ultrapure water with stirring. Adjust the pH to 5.2 with glacial acetic acid; once dissolved, bring the volume to 100 ml. Filter the solution using a 0.2-μm filter membrane. Store up to 3 years at room temperature.

Tris/acetate/EDTA (TAE) buffer, 1×

242 g Tris base
57.1 ml glacial acetic acid
100 ml 0.5 M EDTA solution, pH 8.0
Adjust volume to 1 L for 50× TAE
Dilute 20 ml of 50× stock into 980 ml ultrapure water to make 1× TAE
Store up to 1.5 years at room temperature

COMMENTARY

Background Information

The emergence of hypervirulent *K. pneumoniae* (hvKp) strains has become a significant global concern in recent years. These strains can cause severe community-acquired infections in otherwise healthy people and display enhanced virulence factors compared to classical *K. pneumoniae* strains (Choby et al., 2020). Despite the prevalence of these strains over the past three decades, the precise molecular mechanisms required for hvKp pathogenesis remain underexplored (Lan et al., 2021). Over the years, the genetic manipulation of *K. pneumoniae* has posed several challenges, including the presence of the thick exopolysaccharide capsule that is associated with hvKp strains and limits its transformation efficiency. Furthermore, the high antibiotic-resistance profiles of clinical isolates from hospital-associated infections can make selecting antibiotic markers a challenge.

Preparation of electrocompetent cells for electroporation

One of the techniques used for genetic manipulation in *K. pneumoniae* is the preparation of electrocompetent cells for electroporation (Bachman et al., 2015). Unlike *E. coli*, *K. pneumoniae* is not yet amenable to chemical transformation. Electroporation therefore serves as a useful alternative method for transforming *K. pneumoniae*. However, the bacterium's thick polysaccharide capsule makes preparing competent cells challenging. Therefore, it is important to note that electroporation requires specialized equipment and expertise, which can be a potential disadvantage in terms of accessibility and practicality in certain laboratory settings.

Isogenic mutagenesis using recombineering

Another technique, targeted mutagenesis using the λ Red recombination system, can be employed in *K. pneumoniae* (Bachman et al., 2015). The pKD46-sp^r plasmid carries the necessary genes (γ, β, and exo) comprising the λ Red recombinase enzymes (Datsenko & Wanner, 2000). These enzymes enable efficient homologous recombination, allowing researchers to quickly introduce specific genetic modifications, deletions, or insertions into the *K. pneumoniae* genome. The advantage of the pKD46-sp^r recombinase system is its ability to quickly generate mutants. However, a potential drawback is the possibility of off-target effects with λ Red recombinase, which is why complementation and validation of a mutant are necessary. As the cost of whole-genome sequencing continues to drop, it will likely become common practice to perform whole-genome sequencing to ensure that no off-target mutations are introduced by λ Red recombinase.

Validation of a mutant and verification of the absence of off-target effects

To validate a mutant and verify the absence of off-target effects, a combination of colony PCR and whole-genome sequencing to identify any secondary mutations can be employed. Colony PCR is used for rapid screening of individual bacterial colonies to confirm the presence or absence of the desired genetic modification. Although it offers high throughput and is quick, it cannot detect potential off-target effects. DNA sequencing, on the other hand, provides accurate nucleotide-level information and can identify specific genetic modifications, but it is more time- consuming and costly. Therefore, a combination of these techniques can be employed whereby colony PCR is used for initial validation and then sequencing is performed to verify the absence of off-target mutations. This approach ensures a comprehensive assessment of the mutants while balancing efficiency and accuracy.

Generating clean knockouts using the FLP recombinase

To generate clean knockouts, the FLP recombinase system is utilized. This approach involves the use of the pFLP-hyg plasmid containing the FLP recombinase (Chen et al., 2021; Huang et al., 2014). The pFLP-hyg plasmid enables the excision of the kanamycin selection marker from the chromosome via flippase recognition target (FRT) sites. This specific excision ensures that any observed changes or phenotypic alterations can be solely attributed to the loss of the targeted gene, enhancing the reliability and accuracy of experimental results. The advantage of using pFLP-hyg for generating clean knockouts lies in its ability to perform highly targeted and specific excision events through site-specific recombination at FRT sites, effectively minimizing the risk of unintended mutations or genetic rearrangements. However, one potential drawback of this system is the additional time required to remove the plasmid from the bacterial population. Alternatively, the allelic exchange system can be used to generate clean knockouts, but this may require additional time and expertise.

Complementation vector for phenotypic restoration

Complementation experiments are crucial for studying the relationship between gene mutations and phenotypic changes. Complementation makes it possible to understand the impact of gene mutations or deletions on a phenotype of interest. By introducing a functional copy of the mutated or deleted gene, complementation restores normal gene function and allows assessment of its associated phenotype. These experiments establish a causal relationship between the gene of interest and observed phenotype. One potential disadvantage of complementation is the possibility of overexpression or misexpression of the gene of interest.

Critical Parameters and Troubleshooting

Bacterial transformation via electroporation

When preparing competent cells for transformation, there are several potential errors

to be aware of that can impact the efficiency of the transformation. Monitoring bacterial growth during the mid-log phase is crucial, as exceeding this phase can result in reduced transformation efficiency. Maintaining the competent cells on ice throughout the process is essential to ensure high efficiency. Adequate washing of the cells is important to remove potential salts that could cause arcing during transformation. If the transformation efficiency is poor, it could be due to low-quality DNA. Furthermore, if both the positive control and the experimental control show no growth, this indicates a problem with the viability of the cells. If growth is observed on the negative control, that indicates contamination of the reagents.

Isogenic mutagenesis using pKD46-sp^r

When generating isogenic mutants in *K. pneumoniae* using the λ Red recombinase system, there are potential pitfalls to consider. If PCR product amplification fails, check primer design for errors, ensure DNA template and primers are of good quality, and optimize PCR conditions. The addition of 3% DMSO can help reduce nonspecific amplification. If no colonies grow on selection plates after transformation, confirm antibiotic concentration and plate quality, try increasing DNA concentration, check for contamination during plating, and remember to add L-arabinose during electrocompetent preparation for successful gene excision with the pKD46-sp^r plasmid.

Generating a clean knockout using the pFLP-hyg plasmid

When generating clean-knockout mutants in *K. pneumoniae* using the pFLP-hyg plasmid, there are several potential pitfalls to consider. Insufficient passaging of the colonies can lead to incomplete loss of the pFLP-hyg plasmid. To address this, it is crucial to continuously streak the colonies on low-salt LB at 42°C to promote plasmid loss. If growth is observed on the low-salt LB-hygromycin plate, further streaking and incubation on antibiotic-free LB agar plates is necessary to ensure complete removal of the plasmid. Additionally, accurate selection of colonies that are sensitive to both hygromycin and kanamycin is important. During DNA extraction, proper boiling of PCR tubes and correct thawing of samples are essential for obtaining high-quality DNA templates for sequencing.

Constructing a complementation vector

If amplification of the pACYC184 backbone and gene of interest is not successful, troubleshooting the PCR conditions and primer design may be necessary. Inadequate transformation or ligation can be addressed by optimizing the reaction conditions and adjusting the DNA concentrations. If the complementation vector is not validated through restriction digestion or sequencing, repeating the procedure or considering alternative vector designs can remedy molecular biology challenges.

Understanding Results

Electrocompetent cells

The anticipated result from following the protocol for preparing electrocompetent cells is the production of a suspension of electrocompetent cells highly capable of taking up foreign DNA through electroporation. These cells can be stored at −80°C and used for various molecular biology applications, such as introducing plasmids, gene knockouts, or other genetic modifications into the *K. pneumoniae* strain.

Transformation via electroporation

After completing the protocol for transforming *K. pneumoniae* using electroporation, one anticipated result is growth of colonies on low-salt LB-antibiotic selection plates. These colonies represent the successful transformation of *K. pneumoniae* cells with the desired plasmid DNA. Additionally, growth on a positive control plate indicates the competent cells are efficient. If there is no growth on a positive control plate, this indicates the competent cells are no longer viable or electroporation did not work. If there is growth on a negative control plate, this indicates potential contamination of reagents or the antibiotic in the plate is inadequate. The visualization of colonies serves as a tangible indication of the transformation success and provides researchers with the basis for further genetic studies and applications involving the genetically modified *K. pneumoniae* strains.

Creation of an isogenic mutant

Upon completing this protocol, the anticipated results include the successful generation of isogenic mutants in *K. pneumoniae* with an antibiotic-resistance cassette. The first expected result is visualizing the kanamycin cassette during PCR amplification using gel electrophoresis. Our lab typically

observes 75% to 100% correct insertion of the antibiotic cassette in surviving colonies. Subsequently, the presence of colonies on the low-salt LB-kanamycin plates confirm the successful transformation and integration of the kanamycin cassette into the target gene site. The mutants can be stored in cryovials at −80°C for long-term preservation and future use.

Colony PCR

After a colony PCR screen is conducted, the anticipated results involve detecting the presence or absence of the desired genetic modification through gel electrophoresis. The modification can be identified by comparing the size (in base pairs) of the PCR product with that of the wild-type strain lacking the modification. Restriction digest can further confirm insertion of an antibiotic cassette. This visual comparison allows researchers to determine if the desired genetic modification has been successfully introduced into the target organism.

Validating absence of secondary mutations

Upon completion of this protocol, the anticipated result is a compilation of all locus variants, including the capsule locus. This will identify any undesired secondary mutations within the strain, ensuring a clean genetic modification.

Removal of antibiotic selection marker

After generation of a clean-knockout mutant utilizing the pFLP-Hyg plasmid, the anticipated result would be a gene-deletion strain with no antibiotic cassette. As passaging continues, the beginning of loss of the pFLP-Hyg plasmid can be seen as intermediate sensitivity that makes the colonies appear blotchy on LB-hygromycin agar plates. Upon successfully completing this protocol, the anticipated visual result would be no growth on the low-salt LB-hygromycin (100 µg/ml), no growth on the low-salt LB-kanamycin (25 µg/ml), and growth on the low-salt LB plate.

Complementation vector

After the construction of a complementation vector is completed, the anticipated result would be the restoration of a mutant phenotype, thereby confirming the significance of the gene or genetic element of interest. After PCR amplification of the vector backbone, promoter, and the gene of interest, the anticipated result would be identification of the correct sizes (in base pairs) of PCR products by gel electrophoresis. Upon completion of the ligation and transformation, the anticipated result would be visualization of colonies on a low-salt LB-antibiotic selection plate indicating that the plasmid ligation and transformation was successful. Additional anticipated results would be correct band sizes after a restriction enzyme digestion, sequencing results with no mutations, and restoration to wild-type levels of a mutant strain carrying the complementation vector.

Time Considerations

Preparing electrocompetent K. pneumoniae cells (Basic Protocol 1)

Two overnight incubations are required to have a culture ready for generating electrocompetent cells. The actual electrocompetent cell preparation may take up to 6 hr depending on how long the bacterial culture takes to reach mid-log phase.

Preparing electrocompetent pKD46-sp^r K. pneumoniae cells (Alternate Protocol 1)

The actual electrocompetent cell preparation may take up to 6-7 hr depending on the time it takes the pKD46-sp^r to reach mid-log phase when growing at 30°C.

Transforming K. pneumoniae utilizing electroporation (Basic Protocol 2)

The estimated total time for this protocol is ∼24 hr from start to finish. This duration accounts for the overnight incubation of the plates to allow the colonies to grow and be visible; the transformation using electroporation will take 3-5 hr. This timeframe includes preparing the plasmid DNA and cells, electroporating, recovering, and plating the bacteria. Keep in mind that the actual time may vary depending on the researcher's efficiency and familiarity with the protocol.

Constructing isogenic mutants in K. pneumoniae using the λ red recombinase system (Basic Protocol 3)

Much of the time required for this protocol is for the preparation of electrocompetent cells and PCR product. The estimated total time to complete the entire protocol is 2 days.

Confirming a knockout via colony PCR (Support Protocol 1)

Much of the time required for this protocol is the overnight culture. The estimated time taken to complete the entire colony PCR portion is ∼6 hr.

Verifying absence of secondary capsule locus variants (Support Protocol 2)

Uploading the whole genome sequence file to BV-BRC can take several minutes, whereas the genome analysis may take a few minutes to several days to complete, depending on the number of variants, server availability, and genome size.

Generating clean-knockout mutants in K. pneumoniae using the pFLP-hyg plasmid (Basic Protocol 4)

Much of the time required for this protocol is for multi-day passaging, which takes ∼15 min per day to passage. The estimated time taken to complete the protocol ranges from 5 to 11 days.

Constructing a complementation vector for phenotypic restoration in K. pneumoniae

The estimated total time for this protocol is ∼4 days from start to finish.

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Author Contributions

Brooke E. Ring: Conceptualization; methodology; supervision; writing—original draft; writing—review and editing. **Saroj Khadka:** Writing—original draft; writing—review and editing. **Drew A. Pariseau:** Writing—original draft; writing—review and editing. **Laura A. Mike:** Conceptualization; methodology; project administration; supervision; writing—review and editing.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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