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🌐 Creating a chromosomal knock-in in ADP1 via overlap PCR

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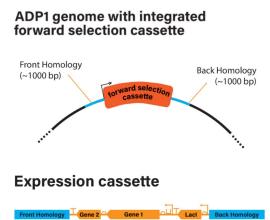
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We use this protocol and it's working

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Abstract

This protocol will outline the steps needed to integrate gene(s) into targeted regions of the ADP1 genome using overlap PCR techniques. This method acts as a follow up to the protocol "Creating a chromosomal knockout in ADP1 via overlap PCR." This protocol will reference use of Benchling, the cloud based organization software for designing genetic parts.

Materials

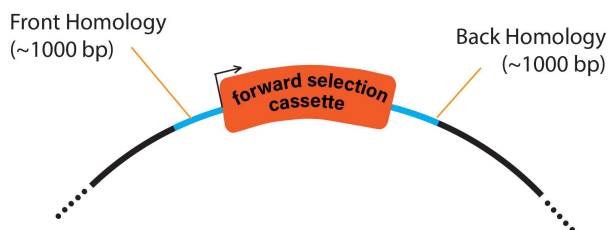
ADP1 ISx strain: <https://www.addgene.org/216551/>.

pBWB162 expression plasmid: <https://www.addgene.org/140634/>.

Protocol overview

- 1
 - This protocol aims to integrate gene(s) into targeted regions of the ADP1 genome (**Figure 1A**). This method acts as a follow up to the protocol "Creating a chromosomal knockout in ADP1 via overlap PCR."
 - To achieve this goal, we use an "expression cassette." The expression cassette is composed of (1) core and (2) outer regions (**Figure 1B**). The expression cassette's core contains the gene(s) for integration under lacI expression control. The expression cassette's outer parts are 500-1000 bp nucleotide sequences homologous to the front and back sequences flanking the targeted integration site.

A: ADP1 genome with integrated forward selection cassette



B: Expression cassette



Figure 1: Overview of expression cassette design. **A.** ADP1 genome highlighting the integrated forward selection cassette the front and back genomic homology regions flanking the cassette. **B.** Schematic for assembly of example expression cassette.

(optional) Gibson assembly of expression plasmid

- 2 **Overview**
 - This section outlines methods to place your gene(s) of interest under lacI regulation.
 - We will use the pBWB162 expression plasmid (<https://www.addgene.org/140634/>) as the backbone, swapping the mCherry gene in the backbone for our gene(s) of interest.

3



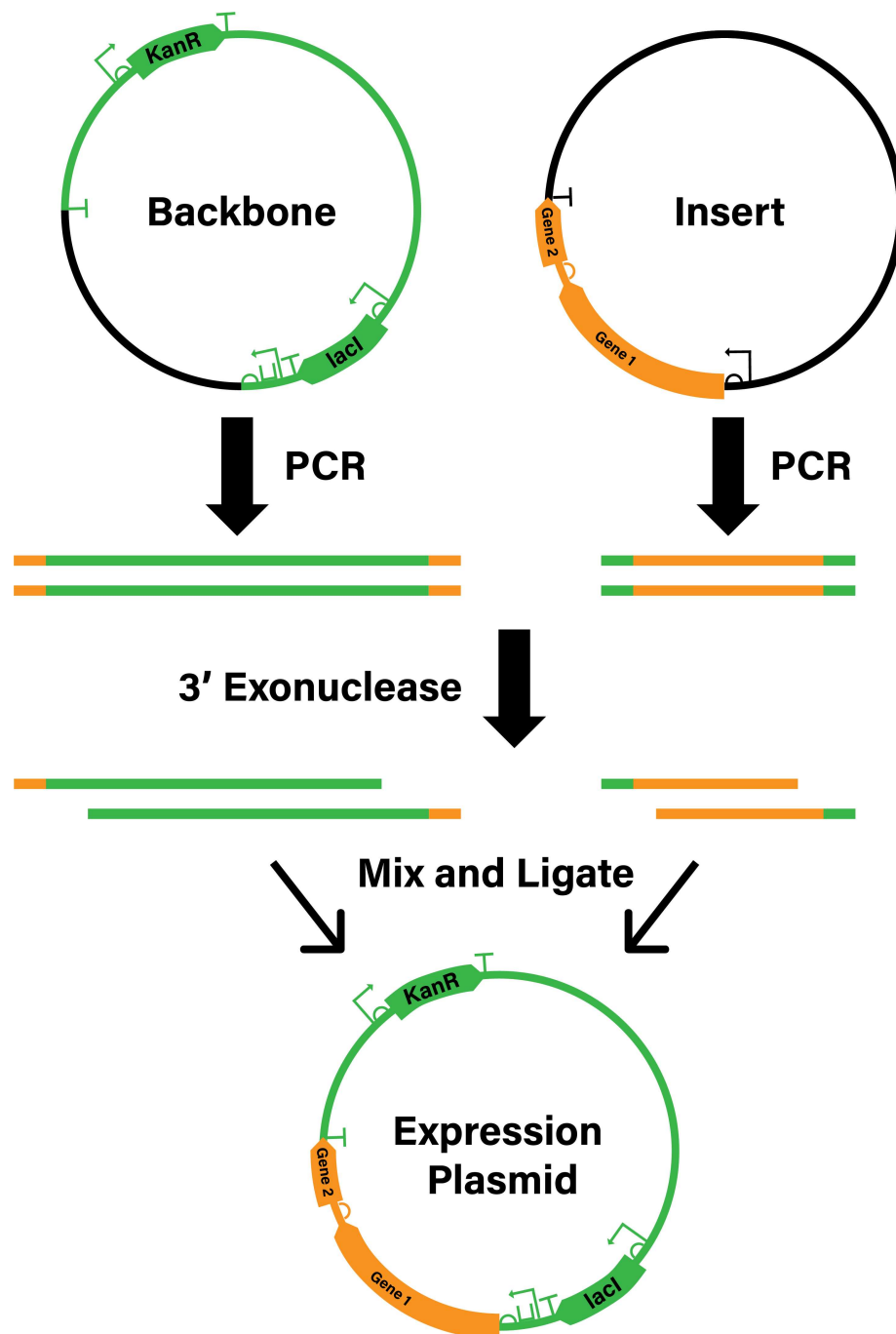


Figure 2: Workflow for Gibson assembly of an expression plasmid.

Quick rules of thumb for designing effective primers

1. Aim for 55° melting temperature.
2. Have the primer start and end with a C or G.
3. Aim for 20-30 nucleotides in length.
4. Aim for total GC content ~40-60%.

5. Avoid regions with repeated strings of any single nucleotide (i.e. five adenines in a row).

Design primers in silico

- Use of a cloning software will streamline Gibson assembly design. Here we utilize Benchling.
1. Design Gibson assembly of parts in Benchling. (Assembly > assembly wizard > Gibson > start > highlight backbone > set fragment > highlight insert > set fragment > *name assembly* > assemble)
 2. Adjust primers if needed (see "*quick rules of thumb for designing effective primers*").
 3. Order primers.

Make ADP1 overnight culture

- A day before you plan to run your PCRs, make an ADP1 overnight culture.
1. Add 5 mL LB to a 14 mL bacterial tube.
 2. Scrape from a glycerol stock or pick a colony from an LB + agar plate and mix into the LB broth.
 3. Grow at 30° overnight.

PCR for Gibson assembly parts and gel extraction

1. Setup 50 µL PCRs for the insert and backbone parts according to your preferred manufacturer's protocol.
2. Amplify PCRs. For PrimeSTAR Max, we use a 10 second extension time per 1 kb product size and amplify for 31 cycles.
3. While the reactions are cycling, pour a 1% agarose + 1x TAE gel.
4. After PCRs complete, add loading dye to each sample and load the entire reaction mix onto the gel. Run the gel until proper separation has been achieved. While the gel is running, label a single 1.5 mL tube for each PCR. These tubes will be used in the next step for gel extractions.
5. Cut the DNA bands from the agarose gel and transfer to the complementary labelled tubes. Use a razor or scalpel and clean the tool between each cut.
6. Purify DNA bands using a DNA extraction kit. Assess DNA concentrations post extraction.

Gibson assembly, transformation into ADP1, and sequence verification of plasmids.

1. Follow the steps in your preferred Gibson assembly protocol/kit. We prefer the "NEBuilder HiFi DNA Assembly" enzyme mix.
2. Transform Gibson assembly product into ADP1. In 1 mL LB media combine 10 µL Gibson assembly product with 70 µL ADP1 culture.
3. Incubate for at least three hours at 30 °C. Post incubation, plate on LB agar + kanamycin (25 µg/mL) selection plate. Incubate overnight at 30 °C.
4. The following day, pick 3-6 colonies. Grow up in 5 mL LB media + kanamycin (25 µg/mL). Incubate overnight.

5. Make glycerol stocks of cultures. Pellet (10 minutes, 4000 x g, 4°). Run minipreps (elute with 35 µL nuclease free water).
6. Check DNA concentrations post miniprep.
7. Send for plasmids for sequencing.

Assemble the expression cassette in silico

4 Overview

- This section focuses on the in silico preparation of the expression cassette (**figure 1B**) using a cloning software. Here we utilize Benchling.

5 Create an in silico map of the expression cassette

1. Create a new Benchling file (Create DNA / RNA sequence > create)
2. Copy the front homology and paste it into the new file.
3. Copy from ~5-15 nucleotides before the lacIq promoter of the expression plasmid assembly to ~5-15 nucleotides after the end of the rrnB T1 terminator. We recommend copying from the sequence "gaactatagctagcatgcatttacg" through "ctagtgtcattttatttcccccgtttc."
4. Paste this region after the front homology.
5. Paste the reverse homology after the expression cassette.



Design primers to amplify the expression cassette

6 Overview

- This section outlines methods to design an overlap PCR (oPCR).
- The expression cassette is synthesized from three independent complementary PCR regions with homologous overhangs: 1. the front homology, 2. lacI regulated gene(s), and 3. the back homology.
- You will design six primers to generate these three parts.
- oPCR primer design uses Gibson assembly like rules to create overhangs (20-40 bp of homology)

7 Design primers

1. Forward primer for upstream genomic flanking region.

1. Pick out a region ~500-600 bp upstream of the junction between the front homology and the forward selection cassette. See note.
2. Pick a span of nucleotides in this region which meets the criteria for primer design (see "Quick rules of thumb for designing effective primers"). Highlight that region and create the primer (Create > primer > forward > *name primer something useful* > save primer).

Note: Designing primers to amplify a longer region of front homology (say 800-1200 bp) will increase downstream integration efficiency; however, larger pieces of DNA put more strain on the oPCR method.



2. Reverse primer for front homology

- This primer will also have a ~20-30 bp overhang complementary with the upstream lacI promoter region.
1. Focusing first on the binding part of the primer, highlight 20-30 nucleotides in the front homology in accordance with the above guidelines for primer design. The highlighted region should end at the junction between the front homology and the upstream lacI promoter (see **Figure 3A**). Create an annotation marking this region. [create > annotation > *name annotation* > save].
 2. Focusing on the primer's overhang, highlight 20-30 nucleotides from the start of the forward selection cassette to a point which meets the above primer design criteria. Create another annotation marking this region (**Figure 3B**).
 3. Create the reverse primer: Highlight both the marked binding and overhang regions, [create > primer > reverse > *name primer something useful* > save primer] (**Figure 3C**).

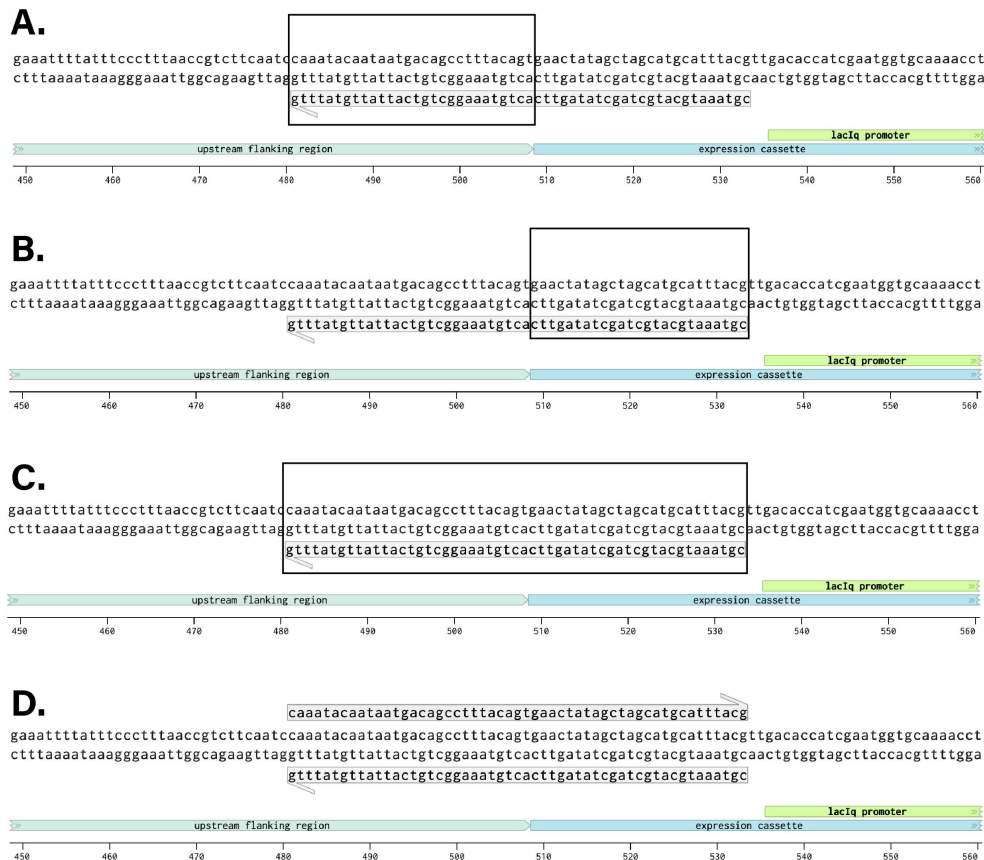


Figure 3: Diagram for designing junction-spanning primers in Benchling. **A.** Box highlighting binding region for front homology reverse primer. **B.** Box highlighting overhang for front homology reverse primer. **C.** Box highlighting complete front homology reverse primer. **D.** Forward primer for lacI regulated gene(s).

3. Forward primer for lacI regulated gene(s)

1. Highlight the two annotations created for the reverse primer.
2. Create a forward primer (create > primer > forward > *name primer something useful* > save primer) (**Figure 3D**).

4. Reverse primer for lacI regulated gene(s)

- Primer design for the second junction mirrors that of the first. Follow the same guidelines outlined above for the previous junction primers.

5. Forward primer for back homology

- Follow the same guidelines outlined for the previous junction primers.

6. Reverse primer for back homology

1. Pick out a region ~500-600 bp downstream from junction between the downstream genomic flanking region and the selection cassette.

2. Pick a span of nucleotides in this region which meets the primer design criteria.
3. Highlight that region. [Create > primer > reverse > *name primer something useful* > save primer].

Order primers from your preferred vender.

PCR amplification of parts for overlap PCR

8 Overview

- Our lab utilizes Takara Bio's PrimeSTAR[®] Max DNA Polymerase (2x master mix) for oPCRs.
- For the front and back homology, we will be using the ADP1 genome ([NC_005966.1](#)) as a template.
- We have found that "colony water" works better than purified ADP1 genome as a PCR template. We provide a method to make colony water below.
- For the expression cassette, we use the expression plasmid outlined in the "Gibson assembly of expression plasmid" section as a template.

9 (Optional) make colony water

- You will need an LB agar plate with individual ADP1 colonies growing on it.
 1. Make colony water: add 30 μ L of nuclease free water to a PCR tube.
 2. Using a pipette tip attached to the pipetter, pick a single ADP1 colony from the LB agar plate.
 3. Transfer the colony to the tube with nuclease free water. Pipette up and down to mix.



Note. The colony water should appear slightly hazy but not opaque.

Run PCRs for overlap PCR parts

1. Setup 50 μ L PCRs according to your preferred manufacturer's protocol. If using colony water, in place of the specified ng DNA, include 1 μ L of colony water/reaction.
2. Amplify PCRs. For PrimeSTAR Max, we use a 10 second extension time per 1 kb product size and amplify for 31 cycles.
3. While the reactions are cycling, pour a 1% agarose + 1x TAE gel.
4. After PCRs complete, add loading dye to each sample and load the entire reaction mix onto the gel. Run the gel until proper separation has been achieved. While the gel is running, label a single 1.5 mL tube for each PCR. These tubes will be used in the next step for gel extractions.
5. Cut the DNA bands from the agarose gel and transfer to the complementary labelled tubes. Use a razor or scalpel and clean the tool between each cut.
6. Purify DNA bands using a DNA extraction kit. We have found that higher DNA concentrations improve success of downstream steps. If you think the extracted band was faint, consider eluting from the gel extraction column with 20 μ L nuclease free

water instead of 35 μL (standard elution volume). Letting the column stand for 5 minutes after adding nuclease free water also helps improve elution efficiency.

7. Check DNA concentrations following extraction.

Prepare overnight ADP1 Δ [gene](::forward_selection_cassette) culture for transformation

- Tomorrow, you can run an overlap PCR and transform the PCR product into your ADP1 knockout strain with a forward selection cassette. Prepare an overnight culture today.

1. Add 5 mL LB to a 14 mL bacterial tube.
2. Scrape from glycerol stock or pick a colony from a prepared LB agar plate, put into the LB media containing bacterial tube, and mix.
3. Grow at 30° overnight.

Running overlap PCR to generate expression cassette

10 Overview

- oPCR uses homology between three PCR products to create a single continuous PCR product.
- The oPCR protocol proceeds through two stages/PCRs: 1. Stage one combines the three PCR products (front homology, lacI regulated gene(s), back homology) and uses the homology regions as primers. This initial PCR runs for 14 cycles. 2. Stage two introduces the front homology forward primer and back homology reverse primer. Addition of these primers encourages formation of the final oPCR product.

11 Setting up and running oPCR

- This work assumes that your front and back homology PCR product concentrations are at least 20 ng/ μL . If you have a lower concentration than this, use at least 20 ng of homology PCR product. It is ok if the sample DNA concentrations are higher than 20 ng/ μL .
- We have found that combining approximate equimolar concentrations of front homology, forward selection cassette, and back homology PCR parts encourages product formation.
- To have sufficient DNA for downstream transformations, it can be beneficial to setup two or three 50 μL oPCR reactions.

1. In PCR tubes, prepare a 50 μL PCRs. In place of manufacturer's specified DNA concentration, add 1 μL each of the front homology, lacI regulated gene(s), and back homology PCR products. Add polymerase/nucleotides/salts. Add water, BUT leave room in the final volume for the later addition of primers during the second stage of oPCR. i.e. if we have a target 50 μL reaction and we plan to add 0.3 μL of each primer in the second stage oPCR, we would add water to our PCRs to a 49.4 μL final volume.



2. Amplify PCRs for 14 cycles under standard PCR conditions. Make sure to adjust specified reaction volume based on the dead-space left in the tube for later addition of primers. For PrimeSTAR Max, We have found that a 10 second extension time per 1 kb product size helps oPCR efficiency.
3. Retrieve the PCR tubes after 14 cycles and add the front homology forward and back homology reverse primers.
4. Return the PCR tubes to the thermocycler. Amplify for 19 additional cycles with the same cycling conditions used during the initial oPCR.
5. While PCRs are cycling, pour a 1% agarose + 1x TAE gel.
6. Once the reactions complete, add loading dye to each sample and load the entire reaction mix directly onto the gel. Run the gel until proper separation has been achieved.
7. While the gel is running, label a 1.5 mL tube for each oPCR. These tubes will be used in the next step for gel extractions.
8. Cut the DNA bands from the agarose gel and transfer to the complementary labelled tubes. Use a razor or scalpel and clean the tool between each cut.
9. Purify DNA bands using a DNA extraction kit. We have found that higher DNA concentrations improve success of downstream steps. If you think the extracted band was faint, consider eluting from the gel extraction column with 20 μ L nuclease free water instead of 35 μ L (standard elution volume). Letting the column stand for 5 minutes after adding nuclease free water helps improve elution efficiency.
10. Check DNA concentrations following extraction.

Transforming ADP1 with expression cassette

12 Overview

- We co-transform ADP1 with the expression cassette and a plasmid which encodes: a Cas9 enzyme, a kanR guide RNA, and a spectinomycin resistance gene. Inclusion of the Cas9 machinery encourages selection for transformed ADP1 strains and enables us to clear ADP1 kanR resistance. Inclusion of the spectinomycin resistance gene enhances selection capacity.
- In our experience, transformed ADP1 cells take a couple days to grow. This most likely occurs due to the Cas9 enzyme's constitutive expression hindering growth.

13 Transforming ADP1 and plating

1. Pellet ADP1 Δ [gene](::forward_selection_cassette) culture. Decant/pipette off supernatant. Resuspend with 5 mL LB. We pellet and resuspend the cells to remove kanamycin from the media prior to transformation.
2. In a 14 mL bacterial tube, combine 1 mL LB, 70 μ L resuspended ADP1 Δ [gene](::forward_selection_cassette) culture, 1000 ng oPCR product, and 500 ng Cas9 plasmid.
3. Incubate for six hours. To our knowledge, this is the optimal amount of time for the transformation. Shorter incubations give lower yields. Overnight incubations also give



lower yields.

4. Plate entire transformation on three LB agar + spectinomycin plates (333 μ L transformation/plate)
5. Incubate at 30 °C for two days.

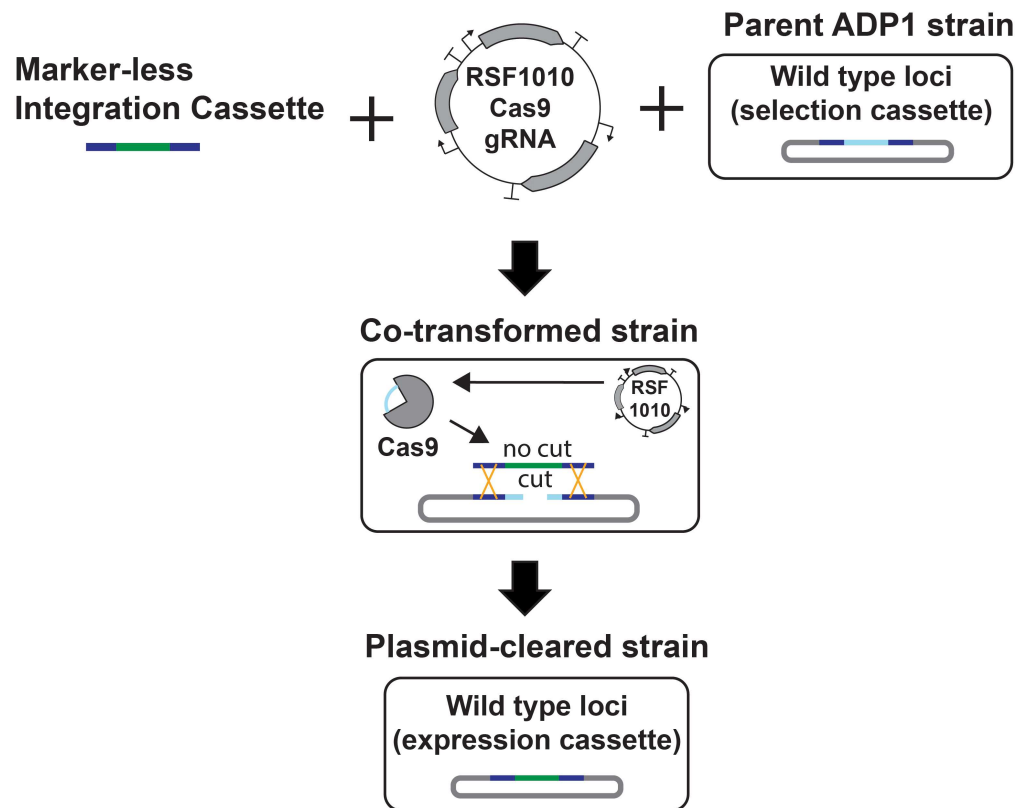


Figure 4: Workflow for transformation of ADP1 with expression cassette and Cas9 plasmid.

Patching, running colony PCRs, and making glycerol stocks

14 Overview

- Creating a stable genomic knockout requires more involvement than integration of the forward selection cassette. Multiple rounds of patching help ensure both the forward selection cassette integrated and the Cas9 plasmid are cleared.

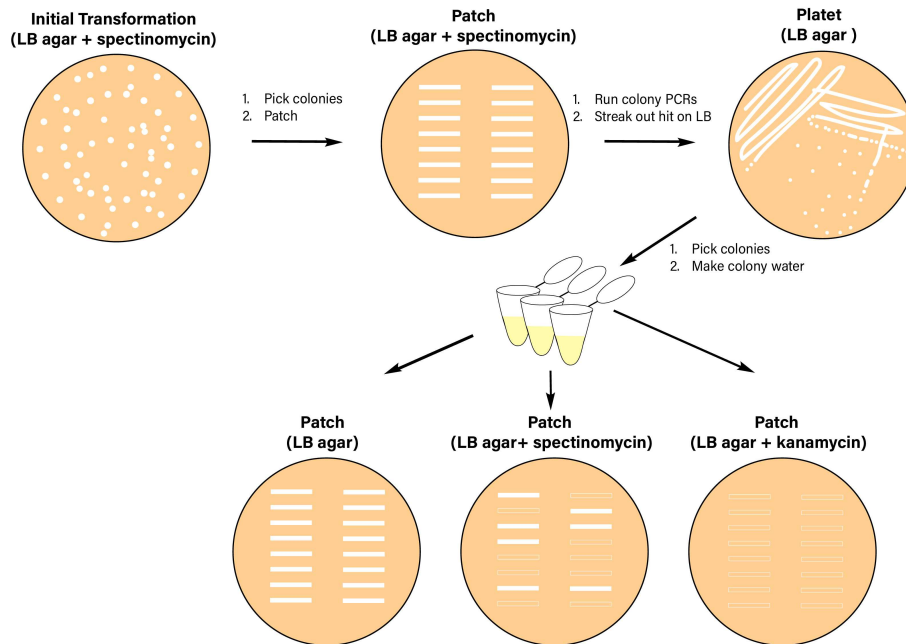


Figure 5: Overview of workflow to establish clonally pure cell line.

15 Patching round 1

1. Check transformation plates for growth.
2. Depending on how many colonies grew, label up to sixteen PCR tubes #1-16.
3. Add 30 μ L nuclease free water to each tube.
4. Pick a colony from the initial transformation plate, put it in PCR tube 1. Pipette to mix. Before ejecting the tip, lightly and briefly streak it on an LB agar + spectinomycin plate. Label the streak with the corresponding PCR tube number.
5. Repeat the process of making colony water and patching with the remaining colonies on the initial transformation plate.
6. Incubate the patch plate overnight at 30 $^{\circ}$ C.

Running colony PCRs from patch plate

- We run colony PCRs (cPCRs) to determine if chromosomal integration occurred. Because these PCR products will not be used for downstream applications, we can run smaller scale PCRs (10 μ L; 9.5 μ L PCR master mix, 0.5 μ L colony water).
1. The following day, check for growth on the patch plate.
 2. Label two sets of sixteen (or whatever number you have of) PCR tubes and number both sets 1-16. The first set should be labelled as colony water. The second set should be labelled as cPCRs.
 3. Add 30 μ L nuclease free water to the colony water labelled PCR tubes.



4. Using a pipette tip attached to a pipetter, lightly tap the edge of the first colony patch growth. Dip the tip into the first colony water PCR tube and pipette to mix.
5. Repeat the process of making colony water for the remaining patched colonies.
6. Create a PCR master mix.
7. Add 9.5 μL master mix to each of the cPCR labelled tubes.
8. In place of DNA, transfer 0.5 μL colony water to each of the corresponding cPCR labelled tubes. We use a multichannel pipette to expedite this process. Do not throw away your colony water! Store it on ice or at 4° for later use.
9. Amplify cPCR reactions following standard PCR thermocycling conditions.
10. While cPCR reactions are amplifying, pour a 1% agarose + TAE gel.
11. Once the samples finish amplifying, add loading dye to each sample and load the entire cPCR mix onto the gel. Run the gel until sufficient separation is achieved. You should see a single bright band at the expected molecular weight.

[If no colonies were positive]

- Cut representative DNA bands from the colony PCR gel, purify, and send for sequencing. This will inform about what might have gone wrong during the transformation.
- You will also need to repeat the oPCR transformation. Try transforming with a greater amount of oPCR product and/or Cas9 plasmid.

Streaking colony water on LB agar plate

1. If you had a positive colony, take 10 μL of the hit's associated colony water and dispense the water onto the edge of an LB agar plate. Streak your pipette tip through the colony water, moving back and forward to cover half of the plate.
2. Eject the tip and get a new one. Lightly drag the new tip through the just-streaked half of the plate and continue to streak the tip across one of the plate's two unmarked quarters.
3. Eject the tip and get a new one. Lightly drag the new tip through the just-streaked quarter of the plate and continue to streak the plate's remaining quarter.
4. Incubate the plate at 30 °C overnight.

Patching round 2

1. Check for single colonies on the LB agar plate.
2. Label sixteen PCR tubes #1-16.
3. Add 30 μL nuclease free water to each tube.
4. Pick a colony from the initial transformation plate, put it in PCR tube 1. Pipette to mix. Before ejecting the tip, lightly and briefly streak it on an LB agar plate, then on an LB agar + spectinomycin plate, and lastly on an LB agar + kanamycin plate. Label each streak with the corresponding PCR tube number.
5. Repeat the process of making colony water and patching with other colonies from the streaked LB agar plate.
6. Incubate the patch plates overnight at 30 °C.



Making glycerol stocks

- We classify a colony as a hit if it grows on LB agar, but not on LB agar + spectinomycin or LB agar + kanamycin (**Figure 5, bottom row**). This would indicate that the chromosomal integration was successful and that the cells have cleared the Cas9 plasmid.
 - If you observe growth on both LB agar and LB agar + spectinomycin for all of your colonies, pick more colonies and try patching again. Alternatively, try patching again from the LB agar patch plate.
1. Assuming you have a hit, Add 5 mL LB to a 14 mL bacterial tube.
 2. Tap a pipette tip into the patch associated with the hit colony and transfer to the LB containing tube. Mix.
 3. Incubate the tube at 30° overnight.
 4. The following day, label a cryovial with strain information and add 0.5 mL 50% sterile glycerol to the vial.
 5. Add 0.5 mL overnight culture to the glycerol, pipette to mix, and store at -80 °C.

Congrats! You have created an ADP1 chromosomal knock-in.

Protocol references

Biggs, B. W., Bedore, S. R., Arvay, E., Huang, S., Subramanian, H., McIntyre, E. A., Duscent-Maitland, C. V., Neidle, E. L., & Tyo, K. E. J. (2020). Development of a genetic toolset for the highly engineerable and metabolically versatile *Acinetobacter baylyi* ADP1. *Nucleic Acids Research*, 48(9), 5169–5182.

<https://doi.org/10.1093/nar/gkaa167>

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