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E. coli recombineering protocol for gene deletions (SW102 prophage protocol)

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We use this protocol and it's working

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Abstract

This protocol is adapted from Thomason et al., 2023 **https://www.ncbi.nlm.nih.gov/pmc/articles/PMC10037674/**

It is written for using the lambda red system (prophage) to make basic gene deletions in E. coli, annotated with practical advice, only for the "Basic Protocol 1" outlined in the source paper.

Guidelines

TIME CONSTANTS

A note on time constants:

the authors originally state that time constant should be above 5.0 ms for maximal efficiency and lower than this is indicative of issues with cells, DNA, or equipment.

Briefly, time constant is the time exposure of the cells to the current, and more allows for better transformation.

Some tricks to increase time constant:

- Keep cuvettes and the part of the electroporator which holds the cuvette and delivers the pulse on ice as cold as possible
- Wipe outside of cuvette with Kimtech immediately prior to delivering pulse
- Stick to smaller volumes of cells and smaller volumes of DNA (if possible).

However, I have had success recombineering in electroporation with time constants as low as 3.6 ms.

Materials

- Electroporator
- Water bath
- Thermometer
- PCR machine
- Benchtop centrifuge with temperature control (benchtop, e.g. Eppendorf 5810-R https://www.eppendorf.com/us-en/eShop-Products/Centrifugation/Multipurpose-Centrifuges/Centrifuge-5810-5810R-p-PF-240994)
- Benchtop microcentrifuge with temperature control
- Cell incubator

CELL LINES

- *E.coli* SW102 cell line, provided by **https://frederick.cancer.gov/resources/repositories/Brb/**
- Alternatively, a plasmid system can be used **https://pubmed.ncbi.nlm.nih.gov/10829079/** but it has not been used in this laboratory

Obtaining your recombineering fragment

1 Amplify a linear DNA fragment for recombineering through PCR. The primers must contain ~50 bp homology arms flanking the region of the genome in to which you wish to introduce your DNA of interest.

Primers which can be used are suggested from the authors below and their sources. For more detailed explanation, see the authors' original paper
https://currentprotocols.onlinelibrary.wiley.com/doi/epdf/10.1002/cpz1.656

Table 4 PCR Primers and Other Suggested Sources of Template for Amplifying Drug Cassettes (Modified from Yu et al., 2000)[a]

Gene	Source	Primer sequence
Ampicillin	T-SACK strain (*bla*)	5′ CATTCAAATATGTATCCGCTC
	pBluescript SK(+) (Stratagene)	
		5′ AGAGTTGGTAGCTCTTGATC
TetA only	T-SACK strain (Tn*10*)	5′ TCCTAATTTTTGTTGACACTCTA
		5′ ACTCGACATCTTGGTTACCG
Chloramphenicol	T-SACK strain (Cat)	5′ TGTGACGGAAGATCACTTCG
	pPCR-Script Cam (Stratagene)	
		5′ ACCAGCAATAGACATAAGCG
Kanamycin	T-SACK strain (Tn*5*)	5′ TATGGACAGCAAGCGAACCG
		5′ TCAGAAGAACTCGTCAAGAAG
Spectinomycin	DH5αPRO (Clontech)	5′ ACCGTGGAAACGGATGAAGGC
		5′ AGGGCTTATTATGCACGCTTAA

[a] It is preferable to use a single-copy insertion on the *E. coli* chromosome as a template (i.e., the T-SACK strain) and the colony PCR procedure for amplification. Although some plasmid templates are suggested for convenience, use of a plasmid template is not recommended.

2 Purify the PCR fragments by gel purification
We use the Qiagen kit with success (QIAEX II Gel Extraction Kit (150) Cat. No. / ID: 20021, https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/dna-purification/dna-clean-up/qiaex-ii-system)

Note: PCR clean up can also be used if the PCR is "clean"

Night before

3 The night before the experiment, incubate SW102 in liquid LB + tetracycline to grow at 30 °C at an appropriate rotation speed.

Note: It is wise to check the temperature of your incubator with a mercury thermometer and calibrate as necessary, as temperatures above 32ºC cause leaky expression of prophage which is deadly to cells if overexpressed. We found that our incubator was around 2.5ºC undershooting the marked temperature and growth was unnecessarily slow.

4 Additionally, autoclave 2 (preferably baffled) 125 mL Erlenmeyer Flasks, and 1 (preferably baffled) 250 mL Erlenmeyer Flask with the opening covered in tinfoil (optional, I have not had contamination problems but it is best to play safe)

Making electrocompetent cells

5 In the 250 mL flask, add 35 mL LB and inoculate with 500 µL of the overnight SW102 culture.

Note: Do not start with a lower dilution than this, although higher dilutions are fine, but will take longer to reach growth.

Leave the culture to grow at 30ºC in a rotating incubator (again, check the temperature with a mercury thermometer - it must not be higher than 32ºC)

6 In the meantime, set a temperature controlled benchtop centrifuge to 4ºC and chill 2 50 mL Falcon tubes.

Set a temperature controlled microcentrifuge to 4ºC.

7 Set a water bath at 42 °C and check the temperature with a thermometer

8 Periodically measure the OD of the growing culture until it reaches OD_{600} = 0.4-0.6.
Note: In practice this occurs at ~2 hours and 45 minutes.

9 When the OD is reached, create a ice-water slurry by mixing ice and water in 50:50 ration.

9.1 Get another ice bucket.

9.2 Then, divide the culture into the 2 autoclaved 125 mL Erlenmeyer Flasks. Label them as induced/non-induced.

10 Leave the non-induced flask in the shaking incubator at 30ºC.

In the water bath at 42 °C (keep the thermometer and check it periodically, adding water if needed or lowering the temperature if needed), shake the induced flask for exactly 15 minutes. Help of a mechanical shaker is useful.

Do not exceed 15 minutes

11 Plunge the induced flask into the slurry and rapidly swirl to cool. Repeat with the non-induced flask.

Leave both flasks on ice for at least 5 minutes.

Additionally, chill a water flask containing at least 100 mL.

12 Every step from now on must be done on ice.

12.1 Spin down both cultures at 4 °C at 4600 x g for 7 minutes.

Decant the supernatant (you can pour)

12.2 Resuspend gently in 1 mL of ice-cold water. Then, add 30 mL of ice-cold water.

12.3 Spin down both cultures at 4 °C at 4600 x g for 7 minutes.

The pellet is extremely soft and WILL resuspend if you tap the bottom of the Falcon tube or attempt to pour it out

Decant the supernatant. **Do not pour. Use a pipettor and aspirate slowly until you have approximately 5 mL. After that, use a 1000 uL pipette until no liquid is left.**

12.4 Resuspend in 1 mL of ice-cold water and transfer to a chilled 1.5 mL tube

Spin down at max speed in the microcentrifuge at 4ºC for 3 minutes.

12.5 Remove supernatant and resuspend in 200 uL of ice cold water. This step can be done more times if desired.

While the cells spin, put at least 3 0.1 mm electroporation cuvettes on ice.

13 Cells are now electrocompetent and can be left on ice up to 2 hours if absolutely needed, but it is best to use fresh.

Electroporation

14 All steps performed on ice.

In clean tubes, mix 50 uL of cells with/without 100 ng PCR product and electroporate. Be sure to carry out the following reactions:

1. Induced + Insert (your desired reaction)
2. Induced + no DNA (selection control)
3. Uninduced + Insert (transformation of background DNA (i.e., plasmid, impure PCR product)
4. Uninduced + known plasmid/fragment as a control for the electroporator working properly.

14.1 Electroporate (press pulse) at 1.8 kV. See the Guidelines on Time constants.

Don't mix DNA and cells first, then electroporate. It is better to do each reaction at a time, followed by the next step, then repeating.

15 Immediately after, add 1 mL of LB to the cuvette, recuperating the cells, and transfer to a 15 mL Falcon and let outgrow at 30 °C for at least 1 hour (recommended 2).

SOC can also be used.

16 After 2 hours have passed, plate on selective media and grow at 30 °C overnight.

Plate at appropriate dilution. In practice, plating 100 µL of the 1 mL of LB culture is sufficient to yield positive recombinants, but overly efficient reactions can cause a lawn. Thus, a 10- or 100-fold dilution can be useful to yield single colonies. In practice, expect an efficiency of 1/500 positive recombinants for ideal conditions.

Verify integration by colony PCR

17 Perform a colony PCR to verify your integration

Removal of prophage

18 The mutant cell line still contains the prophage and it should be removed so it can be grown at 37ºC if no additional mutations are required.

The procedure for removing the prophage is a repeat of the recombineering protocol, but targeting the prophage region for "deletion".

The prophage containing strain is biotin auxotrophic as that is where the prophage is inserted, and thus should not grow in minimal media lacking biotin.

The prophage is deleted by using a linear DNA insert which is obtained from wild-type K12 E.coli strain (MG1655) through colony PCR, using the primers suggested by the authors: The linear fragment is 1.0 kb.

Oligo primers (for amplifying bacterial *attB* site and adjacent *bioA* gene):
5′-GAG GTA CCA GGC GCG GTT TGA TC-3′
5′-GTT GCC GAT GTG CGC GTA CTG-3′

Selection is performed in **M63 biotin- leu+ thr+ plates at 37ºC** in lieu of antibiotic plates.

Prior to plating and after the outgrowth, wash the cells 3 times in M9 media to remove any trace of biotin in the medium. A 1000-fold or higher dilution is suggested prior to plating to disable crossfeeding.

Screen single colonies through colony PCR, using the above primers to verify the loss of prophage (although we had better success using other primers with lower GC content, whose band size is around 1.2 kb):

	A	B
	F.2.bioA.prophage	aagaccgcagagcagagaac

	A	B
	R.2.bioA.prophage	tggtcgaaaccactcatccg

Additionally, verify that the surviving strain also contains your mutation of interest by other colony PCR reaction using appropriate primers.

Protocol references

Thomason, L. C., Costantino, N., Li, X., & Court, D. L. (2023). Recombineering: Genetic engineering in *Escherichia coli* using homologous recombination. *Current Protocols*, 3, e656. doi: **10.1002/cpz1.656**

This is merely a "tips and tricks" from what has been observed in our laboratory after extensive use of the protocol.