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E. coli recombineering (pSIJ8)

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We are still developing and optimizing this protocol.

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Abstract

This protocol is adapted from Jensen et al., 2015 **https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4672327/** for recombineering of gene deletions and posterior excision of antibiotic resistance cassettes using the plasmid pSIJ8

Create your deletion primers

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

Primers should be amplified from pKD3 or pKD4
https://www.addgene.org/45604/
https://www.addgene.org/45605/

Using the priming sites
F 5' tgtgtaggctggagctgcttc 3'
R 5' ctaaggaggatattcatatg 3'

The amplified fragment will contain a selection cassette (Cam for pKD3 or Kan for pKD4), flanked by FRT excision sites.

2 Extract your fragment through method of choice.

Gel extraction works, but PCR cleanup is preferred due to higher yields. Be mindful that PCR cleanup can carry on leftover plasmid template from the PCR reaction and can lead to false positives, so be sure to screen more colonies.

Prepare your strain

3 Transform your desired strain by method of choice with pSIJ8. The below electroporation method works well, ignoring the induction step.

pSIJ8 can be found here
https://www.addgene.org/68122/

This Plasmid contains arabinose inducible lambda red genes for recombineering and rhamnose inducible flippase genes for removal of selection cassette.

Remember that pSIJ8 is temperature sensitive and must be grown at 30C, be mindful of this for the outgrowth step after the transformation too.

Recombineering 6m

4 The night before, start a 10 mL overnight culture of your line hosting pSIJ8 in LB, grow at 30C to saturation with shaking at ~250 RPM.

5 Make a 1:100 dilution of your culture in fresh LB in a sterile autoclaved flask and grow at 30C with shaking at 250 RPM for around 1 hour. A baffled flask is preferable.

6 Measure the OD600 of the culture after 1 hour. It should be around 0.15-0.25

7 Add arabinose at a final concentration of [M] 15 millimolar (mM) . We use a 50X stock for addition of 1 mL in 50 mL of LB.

8 Allow cells to grow for 45 minutes and measure OD. It should be around 0.4-0.6, preferably on the lower end.

9 Pellet cells by centrifugation at 4600 x g, 0°C, 00:05:00 . Discard supernatant. 5m

Keep everything on ice from here on out.

10 Wash with 10 mL 10% sterile ice-cold glycerol 4 times using the same centrifugation speed.

11 After the 4th wash, resuspend in 500 µL ice cold 10% sterile glycerol and transfer to sterile 1.5 mL tube.

12 Pellet the cells by centrifugation at max speed (21300 x g, 0°C, 00:01:00 and resuspend in 500 µL ice cold 10% sterile glycerol 1m

13 Keep cells on ice for 5 minutes.

14 Make fresh transformation tubes - one for each electroporation and aliquot 50 µL cells to each tube.

15 Add your purified DNA product to the transformation tube, briefly flick, and transfer to an ice-cold 0.1 mm electroporation cuvette. https://www.bio-rad.com/en-us/product/gene-pulser-micropulser-electroporation-cuvettes?ID=99a99e78-3336-47f8-805e-67d10397a029

Try to add as little DNA volume as you can, but it should be very concentrated for best success. Aim for ~ 250 - 500 ng.

Only add DNA to the tube that is about to be electroporated. Do not let cell-DNA mixes sit on ice.

16 Pulse at 1.8 kV (Ec1 setting). Record pulse time. Ideally, pulse times of >5 ms are desirable.

17 Immediately add warm 1 mL of LB or SOC to the cuvette and mix to recover as many cells as possible. Transfer to a fresh tube and allow an outgrowth of minimum 2 hours at 30C with shaking.

Make sure the tube is on its side to allow better growth.

18 Repeat steps 14-16 for all remaining electroporations. Make sure you add the additional controls:

- No DNA negative control = for an idea of selection strength
- Uninduced (no arabinose) control with DNA = for an idea of background plasmid contamination electroporation. Especially important if you PCR cleaned-up your fragment.
- The following primer pair can be used as a positive control:
F
GAAGCAGTTAAGCTAGGCGGATTGAAGATTCGCAGGAGAGCGAGCGAAAACTCACGTTAAGGGATTTTG
R
ATCAGCCGGGTGGCAACTCTGCCATCCGGCATTTCCCCGCAAATGGCACTTTTCGGGGAAATGTG

These primers delete the UNG gene and insert the Kanamycin cassette. They do not have FRT sites and amplify well from any pET28 vector.

19 After the 2 hour outgrowth, plate cells in appropriate selection media (**remember to add ampicillin to retain pSIJ8**) and allow them to grow for up to 2 days at 30C.

Removal of the selection cassette

20 Validate your colonies through colony PCR.

21 During the PCR, grow the picked cells in 3 mL LB + amp + kan/cam.

Pour an agarose gel at an appropriate agarose % (suggested 1.2%) to distinguish between positive and negative colonies, as the difference between WT and recombined can be 100-200 bp.

22 After verifying positive colonies on your gel, dilute the cells 1:200 in 50 mL LB + amp (**with no kan/cam**) and with rhamnose (final concentration 50 mM), and grow for around 4-6 hours.

Make sure you include a no-rhamnose control.

23 Plate in LB amp and grow overnight at 30C.

24 Validate your colonies through colony PCR. If the cassette was excised, your band will be shorter than the WT or the unexcised cassette.

25 Proceed to another gene deletion or grow in LB at 37C to remove pSIJ8 from your strain.