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mrdA deletion strain V.1

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Protocol status: Working
We use this protocol and it's working

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Abstract

Making mrdA deletion DH5α strain

- Amplification of PCR product to replace mrdA sequence in the genome (Kan_VcmrdA fragment amplified from pWW308_VcmrdA plasmid). PCR product has homology with EcmrdA sequence at 5' and 3' ends for homologous recombination.
- DH5α strain expressing Lambda Red recombination system proteins from pSIM5 plasmid (introduced into the cells by Heat shock transformation of chemically competent cells - Invitrogen)
-Transform DH5α + pSIM5 cells with Kan_VcmrdA PCR product to replace mrdA sequence in E. coli genome

Materials

- *E. coli* strain DH5α (Library efficiency DH5α competent cells [F^- Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ^-]; Invitrogen)
- pUC19 vector (Supplied with Library efficiency DH5α competent cells; Invitrogen)
- pww308_VcmrdA construct [Tropini C et al 2014]
- pSIM5 plasmid (from Nic Harmer; Lambda Red system; ts origin of replication)
- LB (5g/L NaCl) and SOC medium
- Sterile MQ water
- Sterile 10% glycerol solution
- Oligos (Eurofins)
  CRdelmrdAKanFor and CRdelmrdAKanRev (to amplify Kan_VcmrdA fragment)
  CRdelBFor/CRdelBRev and CRdelCFor/CRdelCRev for colony PCR to confirm deletion
- 30°C/37°Cshaker
- 42°C water bath (Sous Vide Cooking Device)
- Thermomixer
- PCR machine
- Phusion DNA polymerase/OneTaq DNA polymerase (NEB)
- dNTPs (10mM each mix; ThermoFisher)
- Agarose
- TAE 1x
- DNA ladder (GeneRuler 1kb plus, ready to load; ThermoFisher)
- DNA dye (GelGreen 10000x stock in water)
- Centrifuge at 4°C
- 1.5ml eppendorf tubes
- 50ml Falcon tubes
- 0.2ml PCR tubes
- 14ml round bottom culture tubes
- Electroporator (Gene Pulser Xcell, BioRad)
- Electroporation cuvettes (1mm gap)
- Antibiotics
  Kan25 (Kanamycin 25μg/ml)
  Chlr20 (Chloramphenicol 20μg/ml)
  Tet30 (Tetracycline 30ng/ml)
  Amp100 (Ampicillin 100μg/ml)

Transform cells with pSIM5 construct

1

- Streak DH5α cells onto LB agar plate and incubate at 37 °C O/N
- Inoculate LB with one colony from strain DH5α
- Transform chemically competent cells (Invitrogen) with pSIM5 plasmid
- Add 1 µL pSIM5 plasmid to 100 µL DH5α chemically competent cells in a 1.5ml eppendorf tube
- On ice 00:30:00
- Heat shock at 42 °C 40sec (in a water bath) and then incubate the tubes On ice for a couple of minutes
- 900 µL LB to the tube and then incubate at 30 °C Thermomixer 02:00:00
- 3000 x g, 00:05:00
- Remove 900 µL supernatant and resuspend pellet in the remaining media (~100µl)
- Plate cells onto chloramphenicol agar plates [17.5µg/ml]
- Incubate the plates at 30 °C (pSIM has a temperature sensitive origin of replication and will not replicate if cells are incubated at 37°C)
- Pick a colony from the transformation plate and inoculate LB/Chlr17.5
- Incubate culture 30 °C 180rpm Overnight
- Make glycerol stock from the strain
  500µl O/N culture + 500µl 30% glycerol

Note: the stock was transferred directly from ice to -80°C (no flash freezing in liquid nitrogen)

PCR amplification

2 **Primer design**

Amplification of 4169bp DNA fragment from pww308_VcmrdA construct, to replace genomic mrdA sequence.
Primers with ~20nts homology to the plasmid DNA template and ~50nts homology to the genomic target sequence.
Genomic target sequence: 5' and 3' region of E. coli mrdA [first and last 12aa of EcmrdA]

3 **PCR reaction**

Amplification with Phusion DNA polymerase
Buffer HF
dNTP stock 10mM each
Primers: 10pmol/µl each
DNA template 1:1500 dilution in water

Elongation step: 15-30sec/kb [63sec - 126sec] 1min30sec

	5x HF buffer	10
	10m M dN TPs	1
	10pm ol/µl F or pri mer	1
	10pm ol/µl Rev pr imer	1
	Templ ate D NA	1
	Phusi on [2 u/µl]	0.5
	water up to 50µl	up to 50µl

PCR reaction

	Temp eratur e	Time	Cycle s	
	98°C	30sec	1	
	98°C	10sec	30	
	64°C	30sec		
	72°C	1min3 0sec		
	72°C	10min	1	
	4°C	Hold		

PCR fragment purification from agarose gel

4

- Run PCR product on 1% agarose gel

50ml 1xTAE + 1µl 10000x GelGreen stock in water

- Run 5µl from each PCR reaction
- Run 10µl ready to load DNA ladder
- Run gel at 80V 1 hour

DNA purification

- Run the rest of the PCR product on agarose gel - 2x 20µl (2 lanes)
- DNA band cut from agarose gel and purified by column - Monarch Gel extraction kit

[use the B-BOX™ Blue Light LED Epi-illuminator to cut the DNA band from the agarose gel]

Monarch DNA gel extraction kit (NEB)

- Purification of up to 5µg dsDNA from agarose gels
- Add 4 volumes of gel dissolving buffer (100mg agarose band (100µl) + 400µl buffer)
- Each column can take 800µl
- Add ethanol to the wash buffer before using
- Centrifugations at 16000xg RT [Max speed for our benchtop centrifuge is 15000xg]

a. Add 400µl gel dissolving buffer to the 1.5ml eppendorf tube with the agarose gel band (100mg)
b. Incubate in thermomixer at 50°C, 500rpm, for a few minutes until the agarose is completely dissolved (~10minutes)
c. Transfer to the column
d. Spin 15000xg 1minute
e. Discard flow-through
f. Add 200µl wash buffer (with ethanol)
g. Spin 1 minute 15000xg
h. Discard flow-through
i. Repeat the wash with 200µl wash buffer
j. Spin as before and discard flow-through
k. Spin extra 1 minute 15000xg [extra step not in the protocol, to make sure there is no ethanol left in the column]
l. Transfer column to new 1.5ml eppendorf (not supplied)
m. Add 10µl elution buffer and incubate 1 minute RT
n. Spin 15000xg 1 min to elute the DNA

- Mix the purified PCR product from the 2 columns
- DNA stored at +4°C [Purified PCR product eluted in 20µl elution buffer (TE)] until ready to use

DNA quantification

Run 1µl and 3µl purified PCR product on 1% agarose gel (step 8.)
Check if the PCR fragment is correct and ~ DNA concentration

Transform strain eCR106 cells with purified PCR fragment

5 Streak strain eCR106 onto LB /Chlr20 agar plate and incubate at 30°C for ~ 24hours
Inoculate LB/chlor20 with 1 colony from strain eCR106
Incubate the culture O/N at 30°C shaking 180rpm
Check OD of the O/N culture and inoculate 2x50ml fresh LB with O/N culture to get a starting OD of ~0.03-0.05 (250ml erlenmeyer)
No antibiotics added to the cultures
Grow the cells at 30°C 180rpm until OD ~0.4-0.5
Takes ~ 4 hours for the cells to reach the correct density
Mix the 2 cultures and then transfer half of the volume to 42°C 15 minutes (for Red protein induction)
Red protein induction in Sous Vide water bath - keep mixing the cells for the 15 minutes
Keep the other half of the culture at 30°C 180rpm (control: non-induced cells)
Transfer the induced and non-induced cultures to cold 50ml falcon tubes and incubate on ice for ~10 minutes
Centrifuge at 3000xg 10 minutes at 4°C to pellet the cells
Wash cells with 25ml ice-cold water
Resuspend pellet with 1ml ice-cold water. Add the rest of the volume to the tube and gently mix the cells
Centrifuge at 3000xg 10 minutes at 4°C
Wash cells with 15ml ice-cold water
Resuspend pellet with 1ml ice-cold water. Add the rest of the volume to the tube and gently mix the cells
Centrifuge at 3000xg 10 minutes at 4°C
Resuspend the cells in 200µl ice-cold 10% glycerol solution
Aliquot 20µl cells for electroporation in 1.5ml eppendorf tubes
Add ~ 100ng purified PCR product (~2µl) to 20µl electrocompetent cells in an 1.5ml eppendorf tube
Keep the cells on ice

Transform induced and non-induced cells with PCR product
1. Red Induced cells + PCR product
2. Red Induced cells; No DNA added
3. Non-Induced cells + PCR product
4. Non-Induced cells; No DNA added
5. Red-Induced cells + pUC19 (2µl)
6. Non-Induced cells + pUC19 (2µl)

GenePulser XCell protocol
Home screen GenePulser XCell
Pre-set protocols screen
Bacterial protocols
Press Enter to open E. coli 1mm cuvette protocol detail screen

Transfer the cells + DNA to a cold electroporation cuvette
Transport cells/cuvetted on ice to the electroporator (level 3)
Dry the cuvette with blue roll and place the cuvette in the ShockPod
Push the lid down to close
Pulse once
Check time constant
Remove the cuvette from the pod and immediately add 1ml SOC with 10ng/ml tetracycline
Note that this step is not under sterile conditions
Transfer the cells to a 14ml round bottom culture tube and incubate at 30°C 180rpm for 2 hours
Plate transformations onto selection plates:
Transformations 1 and 3:
Plate 100µl onto kan25/Tet30 agar plates
Make serial dilutions up to -6 and then plate dilutions -1 and -2 onto kan25/Tet30 and dilutions -4/-5/-6 onto Tet30 and LB only agar plates
Keep the rest of the transformation at RT on the bench; Plate 100µl onto kan25/Tet30 agar plate after O/N on the bench
Transformations 2 and 4:
Plate 100µl onto kan25/Tet30 agar plates
Transformations 5 and 6:
Plate 100µl onto Amp100 agar plates
Spin the cells and resuspend pellet with ~100µl SOC; plate the cells onto Amp100 plates

Incubate all the plates at 30°C up to 24 hours

Selection of recombinants/Colony PCR

6 Pick colonies that can grow in the presence of kanamycin and re-streak onto new selection plates with kan25
incubate the plates at 30°C up to 24 hours
Pick coloniesfrom the re-streak on kanamycin selection for colony PCR
Pick colony with white loop and resuspend in 20µl water
Use 1µl for PCR reaction
Spot 1µl onto new kan25/Tet30 agar plate and incubate at 30°C up to 24 hours
Colony PCR with primers delB (upstream/downstream EcmrdA sequence) and primers delC (5' and 3' mrdA sequence present in deletion fragment)
OneTaq DNA polymerase
Standard buffer
dNTP stock 10mM each
Primers: 10pmol/µl each
DNA template 1µl resuspended colony in water

Elongation step: 1min/kb; amplification of fragment sizes from 1.2kb to 4.9kb; set elongation to 5minutes

	5x standard buffer	5
	10mM dNTPs	0.5
	10pmol/µl For primer	0.5
	10pmol/µl Rev primer	0.5
	Template DNA	1
	OneTaq DNA polymerase	0.125
	water up to 25µl	17.4

	94°C	30sec	1
	94°C	30sec	30
	49°C	60sec	
	68°C	5min	
	68°C	5min	1
	4°C	Hold	

Run PCR product on 1% agarose gel
50ml 1xTAE + 1µl 10000x GelGreen stock in water
Run 12µl from each PCR reaction
Run 10µl ready to load DNA ladder
Run gel at 80V 1 hour

Set up O/N cultures for the confirmed colonies (LB /Tet) and incubate at 30°C and 37°C (to remove pSIM5 from the cells)
Inoculate media for the O/N culture from the replica plate spots used for colony PCR
Make glycerol stocks
cultures at 30°C - DH5α ΔmrdA + pSIM5 plasmid
cultures at 37°C - DH5α ΔmrdA