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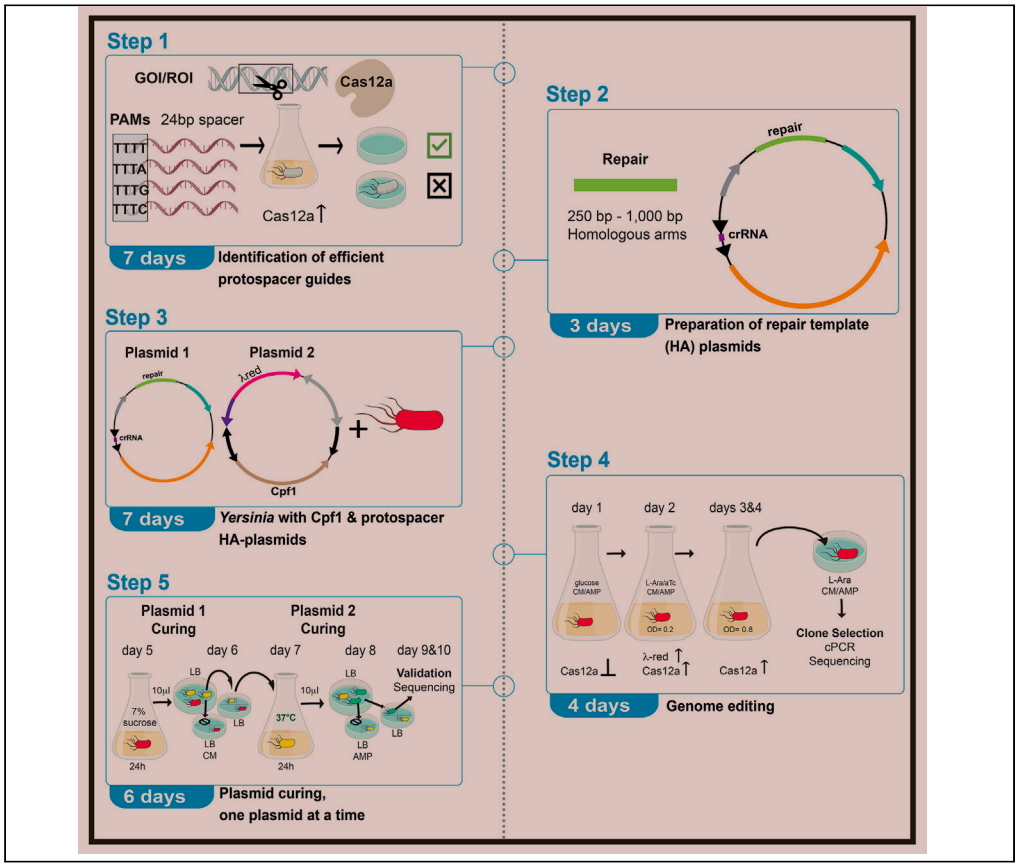
Protocol for genomic recombineering in *Yersinia ruckeri* using CRISPR Cas12a coupled with the λ Red system



Genomic manipulation of *Yersinia ruckeri*, a pathogen of salmonid fish species, is essential for understanding bacterial physiology and virulence. Here, we present a protocol for genomic recombineering in *Y. ruckeri*, a species reluctant to standard genomic engineering, using CRISPR Cas12a coupled with the λ Red system. We describe steps for identifying protospacer guides, preparing repair template plasmids, and electroporating *Yersinia* cells with Cpf1 and protospacer plasmids with homologous arms. We then detail procedures for genome editing and plasmid curing.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

A robust, user-friendly protocol for generating genetic mutants in *Yersinia ruckeri*

Generation of precise gene edits employing CRISPR Cas12a coupled with the λ Red system

Detailed description of plasmid cloning, customizable for varying gene editing needs

Protocol optimized for bacterial species reluctant to standard genome editing techniques

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Protocol for genomic recombineering in *Yersinia ruckeri* using CRISPR Cas12a coupled with the λ Red system

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SUMMARY

Genomic manipulation of *Yersinia ruckeri*, a pathogen of salmonid fish species, is essential for understanding bacterial physiology and virulence. Here, we present a protocol for genomic recombineering in *Y. ruckeri*, a species reluctant to standard genomic engineering, using CRISPR Cas12a coupled with the λ Red system. We describe steps for identifying protospacer guides, preparing repair template plasmids, and electroporating *Yersinia* cells with Cpf1 and protospacer plasmids with homologous arms. We then detail procedures for genome editing and plasmid curing.

BEFORE YOU BEGIN

Swift engineering of genes and genomic regions in pathogen-relevant bacteria, such as *Yersinia ruckeri*, holds immense potential for identification of key pathogenesis factors or resistance islands, accelerating high throughput screening workflows, research, and applications in this field.

Cas12a is a type V, class II single RNA-guided (crRNA) endonuclease CRISPR system, isolated from *Francisella novicida*. Cas12a (gene product of *FnCpf1*), creates 4 or 5 base pair long staggered ends ("sticky ends"), triggering the native SOS repair mechanism of the host cell. Nucleotide resolution precise cutting is mediated by a ~25 base pair crRNA, these short crRNAs allow cleavage of dsDNA next to protospacer adjacent TTTV motives (PAMS). Cas12a generates double strand breaks (DSBs), which can then be repaired by endogenous repair pathways, such as, non-homologous end joining (NHEJ) and homology-directed repair (HDR).[1,2] Both processes result in challenging clone selection; NHEJ is imprecise, resulting in insertions or deletions (indels), whereas HDR is precise, yet clone selection is cumbersome, as homologous arms are heterogeneously included as repair templates, and must be isolated from a mixture in a colony forming unit (CFU).

Here we describe steps for producing large, scarless genomic deletion mutants using CRISPR-Cas assisted recombineering, employing two plasmids (Figure 1), combining the Cas12a system with the λ bacteriophage recombination system, which resulted in precise high efficiency gene editing outcomes. The λ system consists of a 5′-3′ exonuclease (encoded by *redα* or *exo* gene), that binds dsDNA and digests the 5′-strand into mononucleotides,[3] and a single-strand annealing protein (SSAP, encoded by *redβ* or *β* gene) that binds the resulting 3′-overhang and anneals it to a complementary DNA strand.[4]

The first plasmid (pKD46-*FnCpf1*-OP2) encodes the Cas12a CRISPR system as well as the λ bacteriophage recombination system,[5,6] inducible with L-Arabinose and anhydrous tetracycline (aTc), respectively. The tet response element (TRE) is recognized by the tetracycline repressor (tetR). In

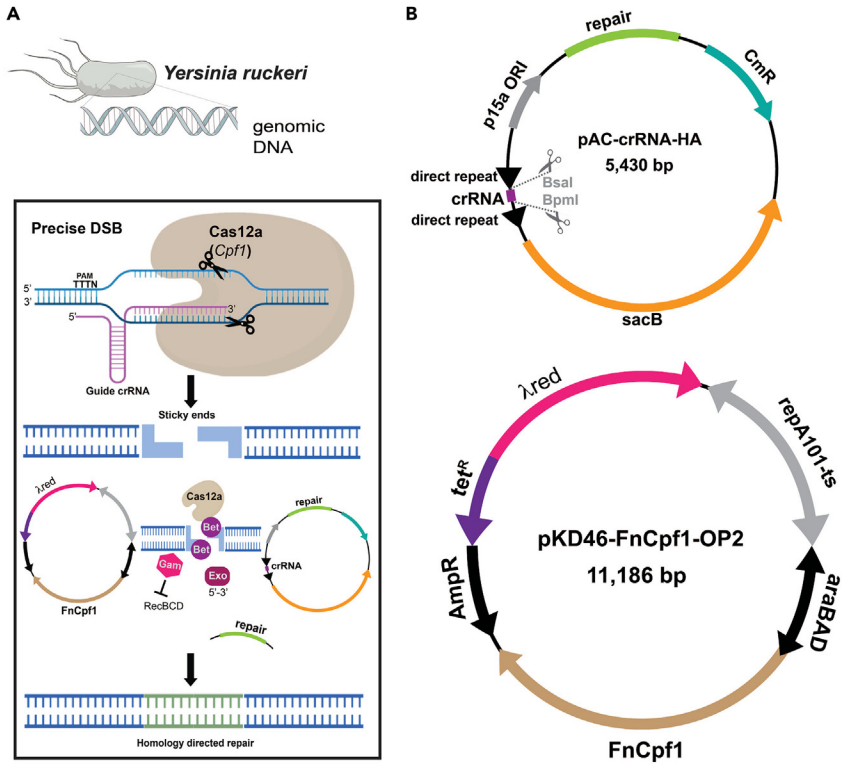

Figure 1. Recombineering in *Y. ruckeri* by coupling Cas12a with the λ Red system and providing the repair template on a plasmid

(A) Schematic describing Cas12a and λ Red system mechanism and components working in tandem.

(B) Plasmid information and important gene features. The pAC-crRNA-HA plasmid (chloramphenicol resistance, CmR) and pKD46-*FnCpf1*-OP2 (ampicillin resistance, AmpR) plasmids used in this protocol. Gene features like antibiotic resistance, origin of replication (ORI), induction features and plasmid curing features (e.g., *sacB*), gene editing elements, Cpf1 and λ Red bacteriophage recombination system, together with the repair template homologous arms (HA) are highlighted. The crRNA guides are flanked by direct repeat sequences and can be inserted using ligation-based protocols (BsaI and BpmI restriction enzymes).

presence of aTc, TetR is bound by aTc lowering its affinity for DNA and allowing for induction of the λ bacteriophage recombination system, under the Ptet promoter (see Figure S1 for details). Ampicillin resistance (AmpR) cassette is used for selection. The pKD46 plasmid harbors replicons oriR101 & repA101-ts (low copy origin of replication), derived from the pSC101 replication origin. Initiation of replication at oriR101 is regulated in trans by the heat-labile protein product of repA101-ts, which loses activity significantly at 37°C (up to 42°C). This temperature sensitive replication mechanism can be used for plasmid curing of the pKD46 plasmid.

The second vector contains pre-crRNA gene cassettes and two direct repeats with restriction sites for introducing crRNA guides, and homologous genomic sequences generated from sites flanking the target deletion,[5] and a p15a origin of replication, low copy number, host range: *Enterobacteriaceae*. The 25 bp crRNA guide and 250 to 1,000 bp homologous arms must be introduced in to the pAC-crRNA plasmid, which codes chloramphenicol resistance (CmR) for selection. The crRNA guides can target any region of interest (ROI) or gene of interest (GOI) on a genome or plasmid. The two plasmids work in tandem to create seamless genomic mutations, whereas their origins of replication are compatible.

Coupling the Cas12a CRISPR system with the λ bacteriophage recombination system, increases homologous recombination efficiency, as compared to Cas9 gene editing systems.[7–9] Furthermore,

shorter (crRNAs) are sufficient for efficient cleavage next to PAMS, and no trans-activating guide RNAs are required. We demonstrate that repair templates on the plasmid have high impact on efficiency, potentially protecting the repair from degradation from exonucleases or end-joining activities,[10] whereas increased recombination frequency enhances genomic alterations using the λ bacteriophage recombination system. Finally, we successfully adapt the protocol to manipulating non-commercial strains of bacteria, paving the way for scarless genomic manipulation in reticent and wild-type genomes.

Experimental design consideration

Any experiments involving modification of microorganisms should be carried out in a GMO-1 safety laboratory. All experiments must be carried out under biosafety requirements including the safe and secure disposal of GMO-1 waste to units appropriate for such waste. Use of appropriate personal protective equipment is advised, followed by thorough decontamination of any waste before disposing it. Access to autoclaving facilities will be required for sterilizing media and materials, and appropriate disposal.

In the following Part 1 and 2 we describe economically viable methods of cloning the required plasmids, alternatively, the crRNAs and homologous arm plasmid can be ordered via (full plasmid) gene synthesis and/or assembled via standard Gibson cloning techniques. All these techniques can be adapted for high throughput requirements.

Detailed gene annotation and sequence of plasmids used in this study is attached as Figure S1. Recombineering Plasmid Details and Features exported from Benchling [Biology Software] (2020–2023) and GenBank files Data S1: Plasmid 1, pAC-crRNA-HA GenBank sequence file, Data S2: Plasmid 2, pKD46-*FnCpf1*-OP2 GenBank sequence file, respectively. Plasmid pAC-crRNA-Cm[7] is available on Addgene (https://www.addgene.org/158711/).

Institutional permissions

Appropriate permissions to modify bacterial genomes will be required in conjunction with GMO-1 laboratory work.

Part 1: Identification of efficient protospacers and preparation of pAC-crRNA plasmids

Timing: 7 days (week plus design, sequencing, and delivery time)

1. Generate guide crRNA sequences for preparation of pAC-crRNA plasmids. Cas12a on the pKD46-*FnCpf1*-OP2 plasmid creates a double strand break with staggered ends after protospacer adjacent motifs (PAM) TTTT, TTTG, TTTA and TTTC. CrRNAs that efficiently create DSBs on the target site will result in cell death noted by few or no CFUs.
   a. Analyze GOI to identify protospacer guides using a preferred software.
      i. Identify 24 bp sequences after PAM motifs, on positive and negative genomic DNA strands.

   Note: For this study, the Benchling electronic lab notebook software for identifying CRISPR cut sites was used.

   b. Procure or generate protospacer guide oligos, along with each oligo's complimentary sequence.
      i. Add sequence modifications (see Table 1), to adhere to staggered sticky ends generated by BsaI or BpmI restriction enzyme digest of the pAC-crRNA plasmids.[7,11]
   c. Digest pAC-crRNA plasmids with BsaI or BpmI enzyme for 1 h at 37°C.
   d. Separate the cut insert (encoding green fluorescent protein [GFP]) from linearized plasmids.
      i. Run digested plasmid mixtures on 1% (w/v) agarose gel.
      ii. Cut out clear band at 4,500 bp and purify.

Table 1. Protospacer guides, complimentary strands, sequence modifications

Cpf1 PAMs	Name	Strand	GC Content percentage	Sequence	Sequence mod (+restriction site in bold)
TTTC	pAC-crRNA-1i	-	70.83%	AGGCCCGACTACCGCCAGACCCGT	**tagat**AGGCCCGACTACCGCCAGACCCGT
		comp			**agac**ACGGGTCTGGCGGTAGTCGGGCCTa
TTTG	pAC-crRNA-2i	-	62.50%	CCCTGGAGCTAACCCTGCGGCTTT	**tagat**CCCTGGAGCTAACCCTGCGGCTTT
		comp			**agac**AAAGCCGCAGGGTTAGCTCCAGGGa
TTTA	pAC-crRNA-3i	-	45.83%	AACTATTCGTTTCAGGCCCGACTA	**tagat**AACTATTCGTTTCAGGCCCGACTA
		comp			**agac**TAGTCGGGCCTGAAACGAATAGTTa
TTTG	pAC-crRNA-4i	+	37.50%	CTTTCCAGAAAGGTATGATTCTGA	**tagat**CTTTCCAGAAAGGTATGATTCTGA
		comp			**agac**TCAGAATCATACCTTTCTGGAAAGa
TTTC	pAC-crRNA-5i	+	37.50%	AGACAGTGAGTCTGTTCATTTAAC	**tagat**AGACAGTGAGTCTGTTCATTTAAC
		comp			**agac**GTTAAATGAACAGACTCACTGTCTa

Pause point: The gel purified and linearized plasmid can be stored at −20°C for a long period of time (years) until further use.

e. Anneal and then ligate protospacers into linearized pAC-crRNA plasmids.
   i. Using either BsaI or BpmI overhangs, in a 1:20 M ratio, incubate ligation mix at room temperature for 5 min.
f. Transform the ligation mix.
   i. Thaw 50 µL of chemically competent *E. coli* 10-beta cells
   ii. Add 7.5 µL of ligated reaction mix to cells and incubate on ice, for 30 min.
   iii. Heat shock cells at 42°C for exactly 45 s, and cool on ice again for 2 min.
   iv. Add 450 µL of LB medium pre-warmed at 37°C, incubate at 37°C shaking at 200 rpm for 1 h.
   v. Centrifuge tubes at 6,000 × *g* for 5 min, decant supernatant to 100 µL, resuspend and plate on LB plates supplemented with chloramphenicol.
   vi. Incubate plates 37°C, 12–18 h overnight.
g. Select and validate protospacer clones:
   i. Select single colonies (4–5 clones) for each protospacer and start overnight growth cultures, incubate 12–18 h at 37°C shaking at 200 rpm.
   ii. Isolate pAC-crRNA-protospacer plasmids from overnight growth cultures, for all plasmids.
   iii. Sequence plasmids to identify correct insertion and orientation of protospacer guides, we sequenced 4 clones for every crRNA sequence (see Table 2).

Pause point: Sequenced and validated protospacer plasmids can be stored at −20°C for long period of time (years).

2. Introduce protospacer guides for guided genomic cutting and for genome editing. Bacterial cells need to be made competent to take up relevant plasmids.

Table 2. Example of crRNA workflow information on annealing, transformation, sequencing result and cutting performance highlighting high efficiency crRNAs

Name	Annealing reaction	Transformation CFU	Positive sequencing clones	crRNAs cutting after electroporation
pAC-crRNA-1i	Done	300 +	1i, 1ii, 1iii, 1iv	1i
pAC-crRNA-2i	Done	300 +	2i, 2ii, 2iii, 2iv	2i
pAC-crRNA-3i	Done	None	-	-
pAC-crRNA-4i	Done	300 +	4ii and 4iv	4ii
pAC-crRNA-5i	Done	10	5ii, 5iii and 5iv	5ii

Note: *Y. ruckeri* cells remain competent at −80°C for up to a year, the amount of medium to seed overnight inoculum into can be increased, if the number of genomic editing experiments planned is larger.

a. Streak out *Y. ruckeri* from glycerol stock on sterile LB plates, work sterile. Incubate plates for 48 h at 26°C.
b. Start growth culture from a single colony on the incubated LB plates in sterile LB medium, for this protocol 1 mL of seed culture was started, incubate 12–18 h at 26°C shaking at 180 rpm.
c. Next day prepare electrocompetent cells.
    i. Inoculate 50 mL of sterile LB medium with 0.5 mL growth culture, with (when preparing induced cells) or without antibiotics.
    ii. If preparing cells for ascertaining protospacer cutting efficiency, induce the Cpf1 system on pKD46-*FnCpf1*-OP2, already transformed into *Y. ruckeri* cells, with 0.2% (w/v) L-arabinose when adding overnight growth inoculum.
    iii. Grow cells at 26°C until they reach OD_{600} 0.6–0.8, ∼ 5–6 h.
    iv. Pellet cells using centrifugation at 5,000 × *g* for 5 min at 4°C.
    v. Decant the supernatant and resuspend the cells in 50 mL of ice-cold sterile ddH_2O and centrifuge same as in step iv.
    vi. Decant supernatant and resuspend the cells in 25 mL of ice-cold sterile 10% (v/v) glycerol and centrifuge same as in step iv.
    vii. Decant supernatant and resuspend the cells in 12.5 mL of ice-cold sterile 10% (v/v) glycerol and centrifuge same as in step iv.
    viii. Decant supernatant and resuspend the cells in 0.5 mL of ice-cold sterile 10% (v/v) glycerol and keep the cells on ice.
    ix. Transfer 50 μL aliquots to prechilled autoclaved tubes suitable for −80°C storage, or electroporate with plasmids immediately.

⚠ CRITICAL: Make sure that all steps after iv. are carried out with ice-cold ingredients and kept on ice until final storage step ix.

Pause point: Electrocompetent *Y. ruckeri* cells can be stored at −80°C up to a year.

3. Identify efficient protospacers by transforming pAC-crRNA plasmids with protospacer guides inserted (pAC-crRNA-protospacer) into electrocompetent *Y. ruckeri* cells, with pKD46-*FnCpf1*-OP2 plasmid induced, using electroporation, pAC-crRNA-GFP can be used as a positive control.
    a. Thaw tubes of electrocompetent *Y. ruckeri* cells, with pKD46-*FnCpf1*-OP2 plasmid induced with L-arabinose (see protocol above) on ice, based on number of protospacer guides, plus one more for control.
    b. Add 100 ng of pAC-crRNA-protospacer or pAC-crRNA-GFP plasmid, incubate on ice for 30 min.
    c. Transfer plasmid cell mix into an electroporation cuvette, keep on ice.
    d. Electroporate using a pulser at 2.5 kV, 25 μF. The time constant should be ∼5.0 ms, no arcing should occur.
    e. Immediately add 950 μL of LB medium prewarmed at 26°C, transfer to a sterile 15 mL culture tube.
    f. Recover for 1.5 h at 26°C, shaking at 180 rpm.
    g. Prepare cells for plates.
        i. Pellet cells by centrifugation at 6,000 × *g* for 5 min in a suitable tube.
        ii. Decant supernatant down to ∼100 μL.
        iii. Resuspend pellet and plate on LB plates supplemented with antibiotics (chloramphenicol and ampicillin).

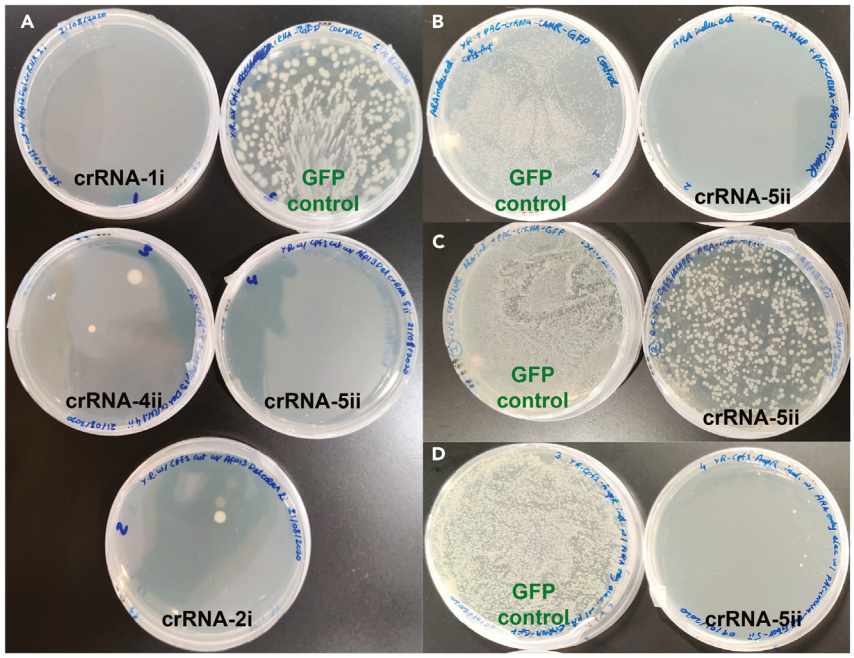

Figure 2. Comparing cutting efficiency of best cutting crRNA guides to the pAC-crRNA-GFP control

(A) A set of good crRNA guides that efficiently cut the GOI/ROI compared to the GFP control (uncut genomic DNA). (B–D) Three independent experiments to show reproducibility of the cutting efficiency (5ii as best crRNA) and obvious difference compared to the GFP control experiment.

iv. Incubate plates at 26°C for 48 h.

h. Compare pAC-crRNA-protospacer plates to the pAC-crRNA-GFP plate. The pAC-crRNA-GFP plates will be a positive control for uptake of the crRNA plasmid by viable cells. A crRNA guide efficient at guiding Cas12a in making a double stranded break, will result in cell death, when this mix is plated, the number of CFUs should be reduced, when compared to the pAC-crRNA-GFP plate. As the number of cells per electrocompetent batch in a tube varies, so will this ratio (see Figure 2).

Pause point: Efficient protospacer plasmids can be stored at −20°C for long period of time (years).

Part 2: Preparation of plasmids with homologous arms (HA)

Timing: 3 days plus sequencing time

A thorough analysis of the GOI/ROI region will determine the length of the homologous arms repair template. For highly repetitive areas or areas with very high GC/AT rich areas we recommend increasing HA region to a maximum of 1,000 bp in total. We recommend starting with two HA repair templates (e.g., 250 bp and 500 bp) if GOI/ROI is surrounded by average GC content and non-repetitive genome patterns.

4. Create seamless deletions, by introducing 250 bp to 1,000 bp homologous arms into the pAC-crRNA-protospacer plasmids.
   a. Use overlap extension PCR (OE-PCR) to generate 250 bp to 1,000 bp homologous arms from each side of the GOI. To do this, two rounds of PCR cycling are employed, generating double stranded DNA arms flanking the GOI, ligated using PCR.

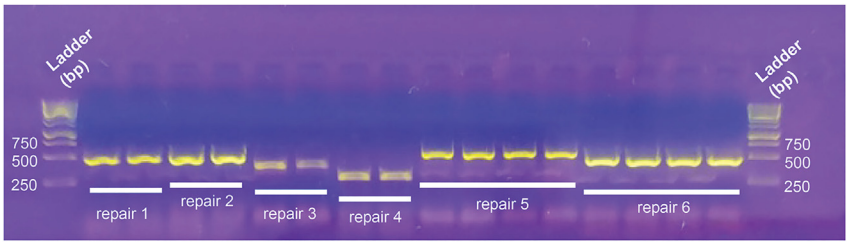

Figure 3. Representative example for OE-PCR results generating various repair templates (repair 1–6)
Bands were cut out, gel purified with high quality kits (Macherey-Nagel NucleoSpin Gel and PCR Clean-up Kit) and DNA concentration measured using the Nanodrop device with concentrations ranging from 12–70 ng/mL.

b. The final product of OE-PCR is generated with overhangs into pAC-crRNA-protospacer plasmids, and gel purified (Figure 3).

Pause point: Gel purified OE-PCR products can be stored at −20°C for long period of time (years).

c. Linearize pAC-crRNA-protospacer plasmids with blunt ends.
d. DpnI digest linearized plasmid to ensure eradication of supercoiled plasmid.
e. Use a ligation independent cloning tool, to fuse homologous arms into linearized plasmid, In-Fusion cloning was used for this study (see materials and equipment 'In-Fusion cloning for introducing homologous arms into pAC-crRNA-protospacer reagents and conditions').
f. Transform 100 ng of constructed plasmid into Stellar competent cells (key resource table) using electroporation, electroporate using a pulser at 2.5 kV, 25 μF. The time constant should be ~5.0 msec, no arcing should occur.
g. Recover cells.
   i. Immediately add 950 μL of LB medium prewarmed at 37°C.
   ii. transfer to a sterile 15 mL culture tube.
   iii. Recover for 1 h at 37°C, shaking at 200 rpm.
h. Prepare cells for plates by
   i. centrifugation at 6,000 × *g* for 5 min in a suitable tube.
   ii. decant supernatant down to ~100 μL.
   iii. resuspend, and plate on LB plates supplemented with chloramphenicol.
   iv. incubate at 37°C for 24 h.
i. Next day, pick single colonies to start cultures in LB medium supplemented with chloramphenicol, incubate at 37°C for 24 h.
j. Next day, isolate pAC-crRNA-protospacer-HA plasmids, and sequence using 1,000 bp Sanger sequencing methods to ensure homologous arms and protospacer sequences have been inserted. Alternatively, or additionally, whole plasmid sequencing can be used to validate the correct insertion of homologous arms.

Pause point: pAC-crRNA-protospacer plasmids with homologous arms (HA) can be stored at −20°C for extensive period (years).

⚠ CRITICAL: For identifying length of homologous regions, within a range of 250–1,000 bp, a detailed analysis of the regions flanking the ROI must be carried out, and HA chosen according to the optimum PCR amplification possibilities (Troubleshooting: Problem 4). Watch out for highly repetitive regions (transposon areas) or regions with high percentage of GC content, which can be difficult to PCR amplify, and require repair template design to vary. *Y. ruckeri* has an optimal growth temperature of 26°C, therefore cells must be grown for 5–6 h to reach optimum electro competency. For efficient genome editing, it is crucial to identify robust protospacer guides, with high cutting

efficiency. It is recommended that a variety of protospacer guides adjacent to differing PAMs (see materials and equipment 'Example of crRNA workflow information on annealing, transformation, sequencing result and cutting performance highlighting high efficiency crRNAs'), to target various sites on positive and negative strands, throughout the GOI, are ordered/generated. Please refer to Figure 1 in Zhao et al. 2018, for details of restriction sites on the pAC-crRNA plasmid series.[11]

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
NEB 10-beta competent *E. coli* *Genotype: Δ(ara-leu) 7697 araD139 fhuA ΔlacX74 galK16 galE15 e14- φ80dlacZΔM15 recA1 relA1 endA1 nupG rpsL (StrR) rph spoT1 Δ(mrr-hsdRMS-mcrBC)*	New England Biolabs	Catalogue #: C3020K
Stellar competent cells part of In-Fusion HD Cloning Plus *Genotype: F-, endA1, supE44, thi-1, recA1, relA1, gyrA96, phoA, Φ80d lacZΔ M15, Δ(lacZYA-argF) U169, Δ(mrr-hsdRMS-mcrBC), ΔmcrA, λ-*	Takara, In-Fusion HD Cloning Kit	Catalogue #: 638911
Chemicals, peptides, and recombinant proteins		
L-(+)-Arabinose 99%, Cas nr. 87-72-9	Thermo Scientific Chemicals	Product Code: 11453904
Anhydrotetracycline	Takara	Catalogue #: 31310
Ampicillin	Sigma-Aldrich	Catalogue #: A9518
Chloramphenicol	Sigma-Aldrich	Catalogue #: C3175
Experimental models: Organisms/strains		
Yersinia ruckeri Ewing et al. 29473	ATCC	CDC 2396-61 [NCIMB 2194]
Other		
DpnI recombinant	New England Biolabs	Catalogue #: R0176L
BsaI HF v2	New England Biolabs	Catalogue #: R3733S
T4 Polynucleotide kinase	New England Biolabs	Catalogue #: M0236L
Macherey-Nagel NucleoSpin Gel and PCR Clean-up Kit	Macherey-Nagel	Catalogue #: 740609.50
Platinum SuperFi DNA master mix	Invitrogen	Catalogue #: 12358010
In-Fusion HD Cloning Plus	Takara	Catalogue #: 638910

MATERIALS AND EQUIPMENT

BsaI digest of pAC-crRNA-GFP plasmid

Reagent	Final concentration	Amount
pAC-crRNA-GFP (41 ng/ μL)	410 ng	10 μL
BsaI enzyme	10 units	1 μL
10 × CutSmart Buffer	1×	5 μL
ddH_2O	N/A	34 μL
Total	**N/A**	**50 μL**

Oligo annealing reaction reagents and conditions

Reagent	Amount	Cycling
Oligo 1 (10 μM)	1 μL	37°C for 30 min
Oligo 2 (10 μM)	1 μL	95°C for 5 min

(*Continued on next page*)

Continued		
Reagent	Amount	Cycling
T4 Polynucleotide kinase (NEB)	0.5 μL	Ramp down to 25 °C at the rate of 5 °C/min
10 × T4 DNA Ligase Buffer (NEB)	1 μL	
ddH_2O	6.5 μL	Total: 10 μL
Total	**10 μL**	

Oligo ligation into pAC-crRNA		
Reagent	Final concentration	Amount
Annealed oligos	380 ng	0.8 μL
BsaI digested vector	20 ng	2.6 μL
Quick T4 ligase buffer (2×)	1×	10 μL
T4 ligase	N/A	1 μL
ddH_2O	N/A	5.6 μL
Total	**N/A**	**20 μL**

OEPCR round 1 reaction master mix	
Reagent	Amount
Genomic DNA	33 ng
SuperFi PCR Master Mix	25 μL
Primer 1 or 3	2.5 μL
Primer 2 or 4	2.5 μL
SuperFi GC Enhancer (5×)	10 μL
ddH_2O	9 μL
Total	**50 μL**

- PCR reactions can be stored overnight at 4°C–6°C in the fridge or for long term at −20°C.

OE-PCR cycling conditions for round 1			
Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	30 s	1
Denaturation	98°C	10 s	25–35 cycles
Annealing	67.8°C and 65.8°C	10 s	
Extension	72°C	20 s	
Final extension	72°C	3 min	1
Hold	4°C	forever	

OE-PCR round 2 reaction master mix	
Reagent	Amount
SuperFi PCR Master Mix	25 μL
Gel purified fragment 1 or 3	2 μL
Gel purified fragment 2 or 4	1.2 μL
SuperFi GC Enhancer (5×)	10 μL
ddH_2O	11.8 μL
Total	**50 μL**

- PCR reactions can be stored overnight at 4°C–6°C in the fridge or for long term at −20°C.

OE-PCR cycling conditions for round 2			
Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	30 s	1
Denaturation	98°C	10 s	15 cycles
Annealing	63.4°C	10 s	
Extension	72°C	20 s	
Final extension	72°C	3 min	1
Hold	4°C	forever	

OE-PCR final round reaction mix	
Reagent	Amount
PCR reactions from round 2	Entire reaction
Primer 1	2.5 μL
Primer 4	2.5 μL

Final cycling conditions for generating homologous arms			
Steps	Temperature	Time	Cycles
Denaturation	98°C	10 s	30 cycles
Annealing	68.7°C	10 s	
Extension	72°C	60 s	
Final extension	72°C	3 min	1
Hold	4°C	forever	

PCR reaction master mix for pAC-crRNA-protospacer linearization	
Reagent	Amount
pAC-crRNA-protospacer	pAC-crRNA-protospacer
SuperFi PCR Master Mix	25 μL
Primer 5	2.5 μL
Primer 6	2.5 μL
SuperFi GC Enhancer (5×)	10 μL
ddH_2O	9 μL
Total	**50 μL**

- PCR reactions can be stored overnight at 4°C–6°C in the fridge or for long term at −20°C.

PCR cycling conditions for pAC-crRNA-protospacer linearization			
Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	30 s	1
Denaturation	98°C	10 s	25–35 cycles
Annealing	69.9°C	10 s	
Extension	72°C	1 min 30 s	
Final extension	72°C	5 min	1
Hold	4°C	forever	

DpnI digest of linearized pAC-crRNA-protospacer plasmid reagents and conditions		
Reagent	Amount	Conditions
pAC-crRNA-protospacer	Entire PCR reaction	37°C for 15 min
10× CutSmart Buffer (NEB)	10 μL	
DpnI (NEB)	10 units	
Total	**N/A**	

In-Fusion cloning for introducing homologous arms into pAC-crRNA-protospacer reagents and conditions		
Reagent	Amount	Conditions
5× In-Fusion HD Enzyme Premix	4 μL	50°C for 15 min
pAC-crRNA-protospacer linearized	150 ng	
250–1,000 bp homologous arms	41.7 ng	
ddH_2O	8.9 μL	
Total	20 μL	

- In-Fusion reactions can be stored long term at −20°C until further use.

STEP-BY-STEP METHOD DETAILS

Part 1: Electroporate *Yersinia* cells with Cpf1 and protospacer HA-plasmids

Timing: 7 days

Prepared electrocompetent cells must be electroporated with appropriate plasmids, while suppressing or inducing the intrinsic Cas12a nuclease activity under the *araBAD* promoter system, with glucose or L-arabinose, respectively. Once the pKD46-*FnCpf1*-OP2 has been transformed into the cells, it is important not to exceed the optimal growth temperature of 26°C, as the plasmid has a temperature dependent self-destruct switch at 37°C–42°C.

1. Add 100 ng of pKD46-*FnCpf1*-OP2 plasmid, to a tube of electrocompetent *Y. ruckeri* cells, rest on ice for 30 min.
2. Add cell and plasmid mix to prechilled electroporation cuvettes, electroporate on a pulser at 2.5 kV, 25 μF. The time constant should be ∼5.0 msec, no arcing should occur.
3. Recover electroporated cells.
   a. Add immediately 950 μL of LB medium prewarmed at 26°C.
   b. Transfer to a sterile 1.5 mL tube.
   c. Recover for 1.5 h at 26°C, shaking at 180 rpm.
4. Plate cells harboring pKD46-*FnCpf1*-OP2 plasmid.
   a. Pellet cells at 6,000 × g for 5 min.
   b. Decant down to ∼100 μL.
   c. Resuspend and plate on LB plates supplemented with ampicillin.
   d. Incubate plates for 48 h at 26°C.
5. Start overnight growth culture from a single colony on the incubated LB plates in sterile LB medium supplemented with ampicillin, for this protocol 1 mL of seed culture was started, incubate 12–18 h, at 26°C shaking at 180 rpm.
6. Next day make the cells electrocompetent same as before, growing to OD_{600} 0.6–0.8 in LB medium supplemented with ampicillin, while repressing the pKD46-*FnCpf1*-OP2 plasmid with 2% (w/v) glucose, and electroporate with 100 ng of pAC-crRNA-protospacer-HA introduced, same as before.
7. Plate cells containing both plasmids on LB plates supplemented with chloramphenicol, ampicillin, and glucose for 48 h at 26°C.

Part 2: Genome editing

Timing: 4 days

⚠ CRITICAL: Genome editing should be carried out immediately after electroporation of pAC-crRNA-protospacer-HA plasmids into the Cpf1 suppressed *Y. ruckeri* cells. For highest efficiency, genome editing should be carried out in culture.

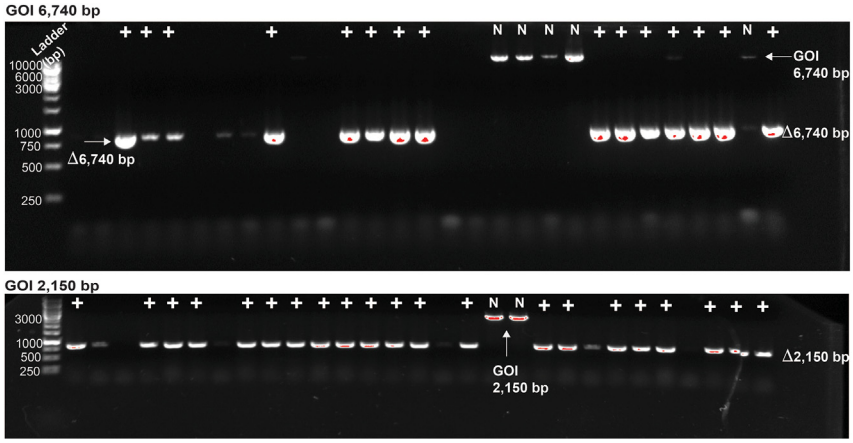

Figure 4. Representative colony PCR gels of positive (+) and negative (N) deletion clones

The gene of interest (GOI) is still present in a few. PCRs were selected to amplify gene of interest (wild type GOI, control), which resulted in both, detection of successful and unsuccessful deletion (GOI presence). We investigated three genomic deletions of various gene sizes, a 2,150 bp, 6,400 bp (toxin), and 6,740 bp (toxin and transposase) bp, resulting in seamless removal with high efficiency (representative results of largest and smallest GOI deletion are shown on top and bottom respectively). Around 100 colonies were tested for each deletion variant and very high efficiency can be achieved with 79% (2,150 bp), 63% (6,740 bp) and 45% (6,400 bp). Percentages were calculated from 100 tested colonies (n = 100) for each recombineering round carried out one time. Positive clones were confirmed with Sanger sequencing and colony PCR (cPCR). Note that efficiency decreased slightly with increasing gene size, and with genomic area. Consider testing more colonies when gene size is increased. Highly repetitive genomic areas can also impact the recombination efficiency.

8. Prepare growth under Cas12a suppression.
   a. Pick a single colony with both plasmids transformed.
   b. Start an overnight growth culture of 1 mL for each genomic mutant, in LB supplemented with chloramphenicol, ampicillin, and 2% (w/v) glucose.
   c. Grow for 12–18 h at 26°C shaking at 180 rpm.
9. Next day, add 0.5 mL overnight growth culture to 5 mL of LB supplemented with chloramphenicol and ampicillin, grow to OD_{600} 0.2.
10. Induce the Cas12a and λ system.
    a. Add 0.5% (w/v) L-arabinose and 80 ng/mL aTc and grow to OD_{600} 0.6–0.8.
11. Grow on plates under Cas12a induction.
    a. Spread 10, 100 and 400 μL on plates supplemented with chloramphenicol, ampicillin, and L-arabinose and incubate at 26°C for each genomic mutant for 48 h.
12. After incubation, check for deletions through colony PCR (Figure 4) with primers 150 bp up and downstream from the 500 bp homologous arms introduced into the pAC-crRNA-protospacer-HA plasmids.
13. Isolate bands from colony PCR and gel purify, to further validate correct deletion with sequencing, such as Sanger sequencing.

Pause point: Edited strains can be stored on plates for up to 2 weeks at 6°C–8°C, or as glycerol stocks at −80°C for months until plasmid curing.

Part 3: Plasmid curing

Timing: 6 days

Note: To continue with functional studies, it is advised to cure both plasmids. For this study (*Yersinia ruckeri ATCC29473*), it was not possible to cure both plasmids simultaneously, by

plating on 7% (w/v) sucrose plates to cure pAC-crRNA-protospacer-HA plasmids (*sacB* counter-selection relies on the toxic product produced by the *sacB* gene from *Bacillus subtilis* that encodes the enzyme levansucrase, which converts sucrose into a toxic metabolite in gram-negative bacteria), followed by incubating at 37°C to cure the pKD46-*FnCpf1*-OP2 plasmid. Instead, they had to be cured individually.

⚠ CRITICAL: Recommended curing temperature is 37°C as *Y. ruckeri* does not survive higher temperatures.

14. Cure pAC-crRNA-protospacer-HA plasmid (Plasmid 1).
    a. Once several deletion clones have been identified, pick a colony from a plate.
    b. Inoculate colonies in LB supplemented with 7% (w/v) sucrose.
    c. Grow overnight 12–18 h at 26°C shaking at 180 rpm.
15. Control pAC-crRNA-protospacer-HA plasmid curing.
    a. Next day, plate on LB only and LB supplemented with chloramphenicol (control).
    b. Incubate at 26°C for 48 h, to ensure plasmid has been cured.
16. Cure pKD46-*FnCpf1*-OP2 plasmid (Plasmid 2).
    a. Pick several colonies from the pAC-crRNA-protospacer-HA cured plate.
    b. Inoculate several overnight LB growth cultures.
    c. Grow for 12–18 h at 37°C shaking at 180 rpm.
17. Control for pKD46-*FnCpf1*-OP2 plasmid curing.
    a. Plate 10 μL from each culture on LB plates supplemented with (control) and without ampicillin.
    b. Incubate at 26°C for 48 h to screen for pKD46-*FnCpf1*-OP2 plasmid being cured.
18. Once a clone with both plasmids cured has been identified, validate genomic mutants with sequencing method of choice.

⚠ CRITICAL: Activation of the λ bacteriophage recombination system using aTc is crucial to obtaining desired clones. Ensure colony PCR primers are at least 150 bp upstream of any repetitive regions flanking the GOI, or the repair templates themselves. *Y. ruckeri* cells will fail to grow in culture or plates at 42°C, whereas the plasmid cured at 37°C.

EXPECTED OUTCOMES

Here we present a genome editing protocol to produce scarless genomic mutants in *Yersinia ruckeri*, which proved reluctant to standard genome editing protocols. Once protospacers, with high cutting efficiency, have been identified, and cloned into the pAC-crRNA plasmid, homologous repair arms flanking the GOI must be generated. Figure 3. is an agarose DNA gel representative of right and left side repair arms. Once these are cloned into the pAC-crRNA-protospacer plasmids, genome editing using recombineering can be carried out. Figure 4. shows representative DNA agarose gels of colony PCR results. Note that PCR cycling for a large GOI may be carried out only for the deleted clone. The presented protocol can substantially improve cloning efficiency and may be of particular interest if comparable methods[4,5] have shown unsuccessful or resulted in poor outcomes.

LIMITATIONS

Limitations of the protocol include the potential for curing the pKD46-*FnCpf1*-OP2 plasmid in bacteria that require strict growth temperature of 37°C or above. The protocol might require adaptions to the antibiotic selection cassette in bacteria with ampicillin or chloramphenicol resistance. Deletion of large and highly repetitive genomic islands can be challenging, but in this study, we addressed this by including deletion of non-essential regions flanking the ROI. Lethal deletions and difficulties with fastidious bacterial strains are additional limitations. Genome cutting efficiency may decrease in the absence of efficient PAMs in the ROI. The protocol theoretically supports insertions or mutations; however, practical testing has not been conducted.

TROUBLESHOOTING

It is important to know the optimal growth temperature, and any resistance already present on the genome. For ROIs/GOIs with repetitive sequences, it is recommended that sequencing is carried out beforehand, especially if the ROI/GOI has been downloaded from a database with shotgun sequencing as it there could be assembly errors. For editing natural plasmids, whole plasmid sequencing is recommended, to correctly identify efficient crRNA guides and homologous repair arms.

Problem 1

Genome cutting and pAC-crRNA cloning issues.

Potential solution

- Opt for PAMs TTTA and TTTT (Table 1), on the negative strand, as these are noted to be more efficient in other studies.
- Try out a variety of crRNA guides (Table 1).
- Transform plasmids for pAC-crRNA plasmid preparations into a plasmid-maintaining cell strain, such as *E. coli* 10β.
- Ensure electro competence efficiency is high by using a positive control plasmid (see Figure 2).
- The pAC-crRNA plasmid is a low copy number plasmid, it is recommended that a preparation of plasmid using a high yield protocol, such as a MIDI-prep is made of pAC-crRNA-protospacer-HA plasmids (sections 4).
- Use excess molar ratio of homologous arms with overhangs into linearized pAC-crRNA plasmids to ensure ligation, as the ratio recommended by the Takara online cloning tool did not result in homologous arm ligation for this study.
- For OE-PCR, gel purifying the PCR reactions to separate homologous arms from incomplete PCR products will result in efficient upstream ligation into the pAC-crRNA-protospacer plasmid.

Problem 2

Yersinia ruckeri or related bacterial strains do not become electrocompetent.

Potential solution

- For bacterial strains that are reluctant to electro competence, consider immediately using small aliquots of cells, instead of using flash frozen aliquots.
- Consider trying a variety of protocols, as electro competence efficiency is highly dependent on optimum growth conditions.
- If strain does not become electrocompetent, consider using transduction/mating protocols to introduce plasmids.

Problem 3

Electrocompetent *Yersinia ruckeri* or related bacterial strains do not take up both plasmids.

Potential solution

- If there was a spark during electroporation, dilute plasmid solution to lower salt concentration, or consider using a plasmid purification step that greatly reduces salt.
- Consider precipitating DNA and resuspending in lower buffer amount to concentrate it.
- Consider using a high yield plasmid purification kit.
- Try electroporation with a range of plasmid concentrations.
- Ensure that recovery media is pre-warmed at 26°C and added to the electroporation cuvette immediately after electroporation.
- Ensure that the time constant is between 4 and 5 m/s.

Problem 4

No clones or clone selection is ambiguous.

Potential solution

- Ensure that a fresh stock of aTc is prepared, for induction of the λ bacteriophage recombination system.
- If there are ambiguous or unexplained bands in colony PCR, consider whole genome sequencing. A combination of short and long read DNA sequencing (Illumina, PacBio, ONT nanopore) will ensure better and correct genomic assembly.
- Re-streak colonies on plates, and repeat colony PCR, in case CFUs contain heterogeneous genomic mutants.
- For genes flanked by repetitive genomic sequences, include larger portions of the repetitive sequence into the homologous arms, up to 1,000 bp.

Problem 5

Curing plasmids is unsuccessful.

Potential solution

- If curing one plasmid at a time does not work, consider curing both at the same time.
- Try curing on plate if curing in culture does not work, or in culture if curing on plates.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Eva Maria Steiner-Rebrova (eva.rebrova@cpr.ku.dk, eva_maria.rebrova@med.lu.se).

Technical contact

Technical requests should be directed and will be fulfilled by the technical contact, Rooshanie Nadia Ejaz (rooshanie.ejaz@cpr.ku.dk).

Materials availability

The generated pKD46-*FnCpf1*-OP2 plasmid with ampicillin resistance and pAC-crRNA plasmids can be shared upon request.

Data and code availability

This study did not generate or analyze any dataset or code.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2024.103014.

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AUTHOR CONTRIBUTIONS

R.N.E. designed and carried out all experiments, with support from E.M.S.-R. and input from N.M.I.T. E.M.S.-R. supported R.N.E. in idea, project planning, and supervision with input from N.M.I.T.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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