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Protocol

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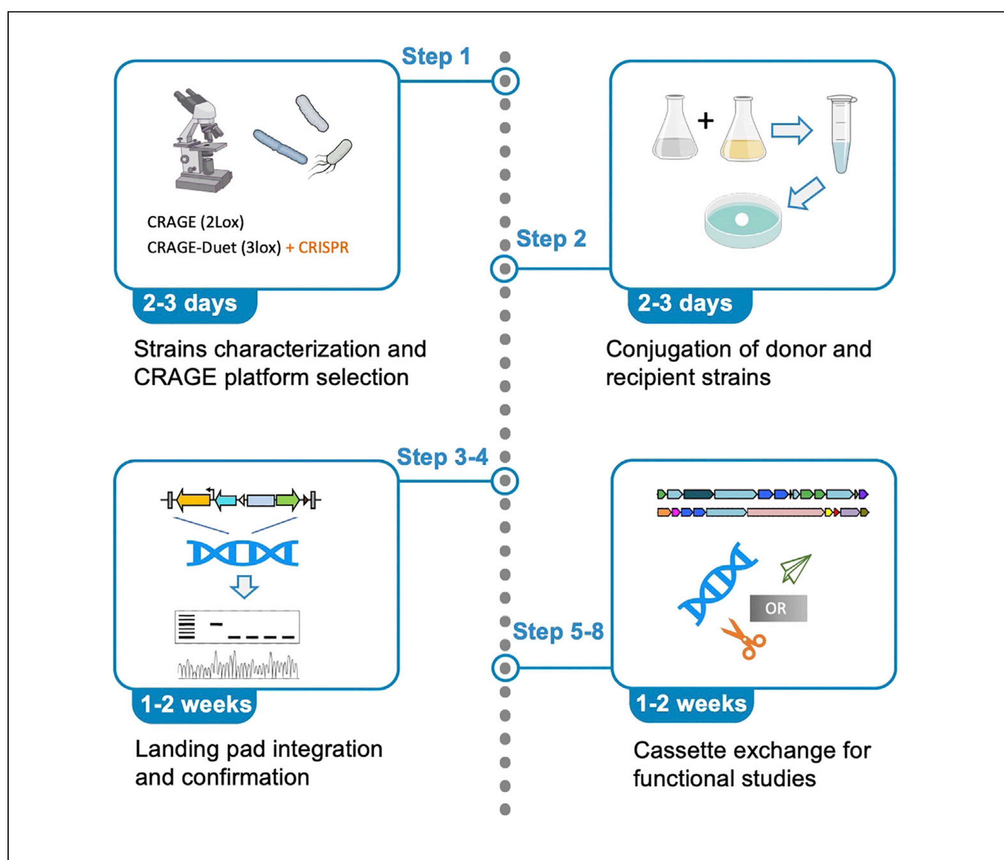

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Highlights

Domestication of non-model bacteria using CRAGE and CRAGE-Duet systems

Single-step chromosomal integration of complex DNA constructs (payloads)

Use of CRISPR-Cas9, CRISPRa, and CRISPRi tools in non-model bacteria

Cas9, CRISPRa, and CRISPRi in diverse bacteria, overcoming major limitations to broaden the application of CRISPR in non-model bacterial genome engineering. Our recent development of the CRAGE (chassis-independent recombinase-assisted genome engineering) system enables single-step integration of large, complex DNA constructs directly into bacteria genomes across multiple phyla. This protocol describes the details of the experimental design and procedures of CRAGE and extended CRAGE-Duet systems. It also describes a strategy that combines CRISPR with CRAGE, which allows implementation of CRISPR-Cas9, CRISPRa, and CRISPRi in diverse bacteria, overcoming major limitations to broaden the application of CRISPR in non-model bacterial genome engineering.

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

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Protocol

Chromosomal integration of complex DNA constructs using CRAGE and CRAGE-Duet systems

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SUMMARY

Our recent development of the CRAGE (chassis-independent recombinase-assisted genome engineering) system enables single-step integration of large, complex DNA constructs directly into bacteria genomes across multiple phyla. This protocol describes the details of the experimental design and procedures of CRAGE and extended CRAGE-Duet systems. It also describes a strategy that combines CRISPR with CRAGE, which allows implementation of CRISPR-Cas9, CRISPRa, and CRISPRi in diverse bacteria, overcoming major limitations to broaden the application of CRISPR in non-model bacterial genome engineering. For complete details on the use and execution of this protocol, please refer to Wang et al. (2019), Wang et al. (2020), and Liu et al. (2020).

BEFORE YOU BEGIN

We recently reported the development of the chassis-independent recombinase-assisted genome engineering (CRAGE) system (Wang et al., 2019). CRAGE enables single-step integration of large complex DNA constructs (payloads) directly into the chromosomes of diverse non-model bacteria. In this technology, we first integrate a landing pad (LP) containing a *Cre* recombinase gene flanked by two mutually exclusive lox sites into the chromosome via an available integration method (e.g., a transposon and a suicide plasmid). We then replace the LP with the payloads (also flanked by the same lox sites), mediated by Cre recombinase; the payloads are inserted with high accuracy and efficiency (Wang et al., 2019). We further extended CRAGE to CRAGE-Duet by introducing a third mutually exclusive lox site, allowing use of any applications a dual plasmid system can offer at the genome level (Wang et al., 2020; Liu et al., 2020).

These CRAGE systems can be ideal platforms for implementing clustered regularly interspaced short palindromic repeat (CRISPR) systems, overcoming a major limitation to using CRISPR in non-model bacteria. CRISPR systems are important tools for functional genomics studies, as gene function can be characterized effectively by deletion and transcriptional repression and activation mediated by CRISPR/Cas9 (CRISPRi), CRISPRd, and CRISPRa, respectively (Gilbert et al., 2013; Peng et al., 2018; Zalatan et al., 2015). Our group implemented CRISPR on CRAGE-Duet and used this approach to characterize secondary metabolite biosynthetic gene clusters (BGCs) using *Photorhabdus luminescens* as a model system (Ke et al. (2021). The results demonstrate that the CRAGE-CRISPR system is a simple yet compelling approach to BGC characterization.

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Table 1. Comparison of CRAGE and CRAGE-Duet systems

	Lox site	1st conjugation (transposon-mediated LP integration) LP plasmid	2nd conjugation (*Cre*-mediated cassette exchange) Accessory plasmid	3rd conjugation (*Cre*-mediated cassette exchange) Accessory plasmid	Pros	Cons
CRAGE	*loxP* and *lox5171*	pW17: the LP containing Km^R and the *Cre* gene flanked by *loxP* and *lox5171* and the T7RP gene under the control of the lacUV5 regulon outside the *lox* sites.	pW34: R6Kr *ori* for pir$^+$ strain, Apr^R and *lux* operon under the control of the T7 promoter flanked by *loxP* and *lox5171*	N/A	A robust system, demonstrated in α-, β-, and γ-*Proteobacteria* and *Actinobacteria*	Only provides one integration site for foreign DNA
			pW5Y: BAC for large payloads, yeast CEN/ARS for TAR cloning, Apr^R flanked by *loxP* and *lox5171*	N/A	Single-step integration of large, complex DNA constructs directly into the genomes of diverse bacteria with high accuracy and efficiency	
CRAGE-Duet	*lox2272*, *loxP*, and *lox5171*	pW37: the LP containing the *Cre* gene flanked by *lox2272* and *loxP* and Km^R flanked by *loxP* and *lox5171*	pW34	pW38: T7RP controlled under the *lacUV5* regulon (inducible with IPTG)	Allows modular integration of two constructs	Some β-*Proteobacteria* strains rearrange the LP to inactivate it. Although unlikely, the mutated *lox* sites could recombine in the presence of Cre, so Cre gene expression should be turned on only when needed
			pW5Y	pW39: T7RP controlled under the *lacUV5* promoter (constitutive)	Multiple editing can be achieved by performing sequential editing	

Here, we describe general protocols for various uses of CRAGE, CRAGE-Duet, and CRAGE-CRISPR systems. These protocols include all the steps necessary to select appropriate CRAGE systems, domesticate non-model bacteria using CRAGE systems, and implement CRISPR systems. We do not include the construction of the LP plasmids, but the plasmids can be requested through our laboratory or purchased through Addgene. While we used a CRAGE-CRISPR system to characterize bacterial secondary metabolite BGCs, we do not include such characterizations in these protocols.

Select an appropriate CRAGE platform for your applications

Timing: ~2 h (user-dependent)

We have developed two different CRAGE systems, CRAGE and CRAGE-Duet. CRAGE enables integration of a single payload, while CRAGE-Duet enables integration of two payloads. To help users select the most appropriate CRAGE system for their applications, this section describes the differences between the two systems and their possible applications (Table 1).

1. CRAGE system.

CRAGE is useful for any applications requiring heterologous gene expression in bacteria. This system uses a plasmid, pW17, to integrate the LP into the recipient bacterial genome. This plasmid comprises a gene coding a mariner transposase and a transposon containing the LP flanked by inverted repeat (IR) sequences. The LP includes a *Cre* recombinase gene and a kanamycin (Km)-resistant gene (Km^R) flanked by two mutually exclusive lox sites (*loxP* and *lox5171*). It also contains a T7 RNA polymerase (T7RP) gene under the control of a lacUV5 regulon (*lacUV5* promoter with *lacI* gene) outside the region flanked by the *lox* sites. This plasmid can be transformed into recipient microbes using conjugation, electroporation, or other methods. Upon transforming pW17, the LP transposons

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integrate at random locations in the genomes of the bacterial population. Users can select a bacterial strain whose phenotype has been least affected by integration for subsequent use.

2. CRAGE-Duet system.

The CRAGE-Duet system offers dual integration sites, allowing any applications that dual plasmid systems enable at the genome level. For example, it allows modular assembly of pathways and introduction of new regulations to orthogonal expression systems (e.g., the T7 expression system). CRAGE-Duet uses the pW37 plasmid to integrate the 3-lox LP into the recipient bacterial genome. This LP contains the Km^R and Cre recombinase gene, flanked by *loxP* and *lox5171* (the first integration site) and *lox2272* and *loxP* (the second integration site), respectively. As with the CRAGE system, this plasmid can be transformed into recipient microbes using conjugation, electroporation, or other methods. Upon transformation, the LP transposons integrate at random locations in the genomes of the bacterial population. Users can select a bacterial strain whose phenotype has been least affected by a particular random integration for subsequent use.

Select appropriate CRAGE accessory plasmids

Timing: ~2 h (user-dependent)

We have developed a variety of accessory plasmids to help integrate payloads into the genomes of target non-model bacteria. This section helps users choose and request suitable accessory plasmids.

We designed the accessory plasmids **pW34** and **pW5Y** to target the CRAGE LP and the first integration site of the CRAGE-Duet LP. The payloads on these plasmids, flanked by two mutually exclusive *lox* sites (*loxP* and *lox5171*), are integrated into the LPs with *Cre*-mediated cassette exchange.

pW34 can be used to confirm that CRAGE or CRAGE-Duet has been implemented successfully. This accessory plasmid carries the *luxCDABE* operon under the control of the T7 promoter between two *lox* sites. Upon transformation of pW34, the *luxCDABE* operon is integrated into the genome of the recipient strain via *Cre*-mediated cassette exchange. Because the payload also contains the apramycin (Apr) resistance gene (Apr^R), simple selection with Apr resistance followed by counterselection with Km sensitivity allows users to identify successful integrants. Detection of luminescence also confirms integration of this payload. After this simple counterselection, integration efficiency is usually 100%. The backbone of the pW34 plasmid can be used as a template for the integration of other payloads. Because pW34 uses R6Kγ as an origin of replication, we recommend using this plasmid to integrate constructs of up to 12 kb.

pW5Y is used to integrate payloads larger than 10 kb. Because this plasmid is a derivative of a BAC-based plasmid, it is cumbersome to use. We recommend using it only when payloads are so large that pW34 cannot stably maintain them. pW5Y also contains yeast CEN/ARS and is compatible with yeast transformation-associated recombination (TAR) cloning to assemble large complex DNA constructs. We found pW5Y tends to replicate in bacterial species (mainly species in *Enterobacteriaceae*) genetically closer to *E. coli*. See potential solutions for problem 4 for troubleshooting.

The accessory plasmids **pW38** and **pW39** are derivatives of **pW34** but are designed to target the second integration site of the CRAGE-Duet system. These plasmids contain T7RP under the control of the *lacUV5* promoter and *lacUV5* regulon. The payloads between two mutually exclusive *lox* sites (*lox2272* and *loxP*) on these plasmids are integrated into the second integration site of the CRAGE-Duet LPs with *Cre*-mediated cassette exchange.

Design the CRAGE-CRISPR system

Timing: 1–2 days (user-dependent)

The versatility of the CRAGE-Duet system allows users to combine it with the powerful CRISPR systems in non-model bacteria for functional genomics studies. This section describes the accessory plasmids required for implementation of the CRAGE-CRISPR system.

The accessory plasmid **pR6K-2L-SpCas9** implements CRISPRi and CRISPR/Cas9, and **pR6K-2L-dCas9-RNAPω** implements CRISPRa. **pR6K-2L-SpCas9** contains the Cas9 gene and the *recET* genes, and **pR6K-2L-dCas9-RNAPω** contains the dCas9-RNAPω gene. In both plasmids, these genes are flanked by *loxP* and *lox5171* and are targeted to the first integration site of the CRAGE-Duet system.

The accessory plasmid **pR6K-lox2272WT** integrates the custom sgRNA and/or repair arm into the second integration site. For the sgRNA and repair arm, users design 20 bp and 500 bp sequences based on the target sequence in the recipient bacterial genome. **pR6K-2L-SpCas9**, **pR6K-2L-dCas9-RNAPω**, and **pR6K-lox2272WT** are derivatives of **pW34**.

In addition to working with CRAGE-Duet, which has a 3-lox LP, CRISPR might also work with the 2-lox-LP CRAGE, in which Cas9, recombinases, sgRNA, and repair DNA template are all included in a single accessory vector.

Choose conjugal donor strain

We used the conjugal donor strain *E. coli* BW29427 to transform all LPs and accessory plasmids. Users can easily select out this strain on LB agar plates without diaminopimelic acid (DAP). All the plasmids we created are compatible with other commonly used *E. coli* conjugal donor strains, including S17-1, SM10 (Simon et al., 1983), and ET12567(pUB307) (Flett et al., 1997), if the user prefers a different strain.

Test antibiotic resistance of target bacteria

Timing: 2–3 days

To facilitate selection of integrants, users must determine the minimal inhibitory concentration (MIC) of several antibiotics for the target bacteria. Our studies found many bacteria cannot grow with Km and Apr concentrations of more than 500 μg/mL or 200 μg/mL, respectively. Therefore, we recommend that users test 50, 200, and 500 μg/L of Km, and 50, 100, and 200 μg/mL of Apr. After overnight liquid culture, users will spot 1 μL of each cell on plates containing antibiotics with different concentrations. Alternatively, they can use antibiotic test strips to determine the MICs.

> *Note:* For strains with a natural resistance of more than 1 mg/mL, users may consider different antibiotic markers to replace the Km in the landing pad and Apr in the accessory plasmids as alternatives.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
TransforMax™ EC100D™ pir+ Electrocompetent *E. coli*	Lucigen/Epicenter	Cat# ECP09500
E. coli BW29427	University of Illinois	#aka WM3064
Chemicals, peptides, and recombinant proteins		
2-log DNA ladder	New England Biolabs	Cat# N0550S
96-well black/clear flat bottom polystyrene NBS microplate	Corning	Cat# 3651
Agarose UltraPure	Invitrogen	Cat# 16500-500
Amino acids mix	Sunrise Science Products Inc.	Cat# 1001

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Apramycin sulfate salt	Sigma-Aldrich	Cat# A2024-1G
Betaine	Sigma-Aldrich	Cat# B2629-100G
$CaCl_2$ solution, 1 M	Sigma-Aldrich	Cat# 21115
Citric acid monohydrate	Sigma-Aldrich	Cat# 1909
D-glucose	Sigma-Aldrich	Cat# G8270
Costar 96-well clear round well plate, sterile with lid	Fisher	Cat# 07-200-760
Diaminopimelic acid	Sigma-Aldrich	Cat# 33240-5G
Filter membranes, nitrocellulose	Sigma-Aldrich	Cat# N8395-100EA
Gel loading dye, purple (6×), no SDS	New England Biolabs	Cat# B7025S
Gene Pulser®/MicroPulser™ electroporation cuvettes, 0.1 cm gap	Bio-Rad	Cat#1652089
Gibson assembly HiFi HC 1-step kit	SGI-DNA	Cat# GA1100-4X10
Glass balls (5 mM)	VWR	Cat# 26396-596
LB agar plate	Teknova	Cat# L1066
LB agar plate with 50 μg/mL apramycin	Teknova	Cat# L1066
LB agar plate with 50 μg/mL kanamycin	Teknova	Cat# L1025
LB agar plate with 200 μg/mL kanamycin	Teknova	Cat# L1077
LB (Miller's) broth	Growcells	Cat# MBLE-7030
Kanamycin sulfate salt	Sigma-Aldrich	Cat# 60615-5G
M9 minimal salts (2×)	Fisher	Cat# A1374401
$MgSO_4$ solution, 1 M	Sigma-Aldrich	Cat# M3409
Phusion High-Fidelity PCR Master Mix (2×)	New England Biolabs	Cat# M0513S
SOC medium	New England Biolabs	Cat# B9020S
Sybr Safe	Invitrogen	Cat# S33102
TAE, 50×	Growcells	Cat# MRGF-4210
Trace mineral solution	ATCC	Cat# MD-TMS
Vitamin supplement	ATCC	Cat# MD-VS
Yeast extract	Sigma-Aldrich	Cat# Y1625
Critical commercial assays		
Gel extraction kit	Promega	Cat# A9282
Plasmid midi kit	Promega	Cat# A2495
Quant-it dsDNA HS assay kit	Invitrogen	Cat# Q32851
DNeasy blood & tissue Kits	QIAGEN	Cat# 69506
Experimental models: Organisms/strains		
Photorhabdus luminescens subsp. *laumondii* TT01	DSMZ	Cat# DSM15139
Pseudomonas simiae WCS417r	JGI	SB119
Serratia odorifera	DSMZ	Cat# DSM4582
Oligonucleotides		
CCACCTTCGTAAGACTGTAGTG (forward primer for CRAGE LP region)	IDT	SBP572
TCCCAGATCTCAAACTGGAACAACACTC (reverse primer for CRAGE LP region)	IDT	99W
GAAAAGCTGGGCGCGTTAAGCC AGCCCCGACACCCG (forward primer for CRAGE backbone region)	IDT	SBP202
GGGGCTGGCTTAACGCGCCCAGCT TTTCAATTC (reverse primer for CRAGE backbone region)	IDT	SBP203
GCTATCAGGACATAGCGTTGG CTACC (forward primer for CRAGE-Duet LP and backbone region)	IDT	197_DT
GTAATGCTCTGCCAGTGTCGCG (reverse primer for CRAGE-Duet LP region)	IDT	190_DT
CGCTGCATAACCCTGCTTCGG (reverse primer for CRAGE-Duet backbone region)	IDT	44W
AGAGTTTGATCMTGGCTCAG (forward primer for 16S)	IDT	27F

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
GGTTACCTTGTTACGACTT (reverse primer for 16S)	IDT	1510R
Recombinant DNA		
pW17: plasmid for CRAGE 2-lox-LP integration	Addgene	Cat# 158207
pW34: accessory plasmid with R6Kγ origin for integration of *luxCDABE* operon (or other payloads) to confirm successful implementation of CRAGE and the first integration site of CRAGE-Duet	Addgene	Cat# 158210
pW5Y: single-copy accessory plasmid for integration of large complex constructs	Addgene	Cat# 158211
pW37: plasmid for CRAGE-Duet 3-lox-LP integration	Liu et al. (2020) and Wang et al. (2020)	N/A
pW38: accessory plasmid containing T7RP under the IPTG-induced *lac* promoter, targeting the second integration site	Wang et al. (2020)	N/A
pW39: accessory plasmid containing T7RP under the constitutively expressed *lac* promoter, targeting the second integration site	Wang et al. (2020)	N/A
pR6K-lox2272WT: base-accessory plasmid targeting the second integration site of CRAGE-CRISPR system	Liu et al. (2020); Ke et al. (2021); this paper	N/A
pR6K-2L-SpCas9: accessory plasmid to implement CRISPR, targeting the first integration site of the CRAGE-Duet system	Liu et al. (2020), Ke et al. (2021); this paper	N/A
pR6K-2L-dCas9-RNAPω: accessory plasmid to implement CRISPRa, targeting the first integration site of the CRAGE-Duet system	Ke et al. (2021); this paper	N/A
Software and algorithms		
Plasmid DNA virtual map, manipulation, and primer design	Geneious Prime	N/A
Integrative Genomics Viewer (IGV)	Broad Institute	N/A
Other		
Gene Pulser Xcell™ electroporation system	Bio-Rad	165–2660/68
Synergy H1 microplate reader	BioTek	N/A

MATERIALS AND EQUIPMENT

Prepare buffers, plates, and media in advance and store them at 4°C to extend their shelf life. Store all antibiotics at −20°C to extend their shelf life. We recommend handling all bacterial cultures in a laminar flow cabinet to maintain sterilized conditions. Users should follow their institutions' protocols for waste disposal, and should properly bleach or autoclave all biological wastes before disposal.

M9-based media		
Reagent	Final concentration	Amount
Glucose	4 g/L	4 g
Yeast extract	5 g/L	5 g
2× M9 minimal salts	1×	500 mL
Citric acid monohydrate	3 g/L	3 g
1 M $MgSO_4$ solution	2 mL/L	2 mM
1 M $CaCl_2$ solution	100 μL/L	0.1 mM
Trace mineral solution	2.5 mL/L	2.5 mL
Vitamin supplement	2.5 mL/L	2.5 mL
ddH_2O	n/a	493 mL
	Total:	1000 mL

Note: Sterilize the media using a 0.22 μm filter, and store it at 4°C for up to 1 year.

Agarose gel		
Reagent	Final concentration	Amount
Agarose	1%	1 g
1× TAE	1×	100 mL
Sybrsafe (10,000×)	1×	10 μL
	Total:	**100 mL**

⚠ CRITICAL: EDTA in TAE may cause respiratory tract irritation. Use a mask to avoid breathing dust. Wear protective gloves, eye shields, and clothing. If there is any skin or eye contact, wash with water for several minutes.

Betaine		
Reagent	Final concentration	Amount
Betaine	5 M	29.275 g
ddH_2O	n/a	50 mL
	Total:	**50 mL**

Note: Prepare the stock solution at 5 M, sterilize it using a 0.22 μm filter, store it at −20°C, and use it at 0.5 M final concentration in PCR reaction.

Alternatives: Users may use 5% DMSO as an alternative to Betaine.

Diaminopimelic acid (DAP)		
Reagent	Final concentration	Amount
Diaminopimelic acid	60 mM	114 mg
ddH_2O	n/a	10 mL
	Total:	**10 mL**

Note: Prepare stock solution at 60 mM, sterilize it using a 0.22 μm filter, store it at −20°C, and use it at 0.3 mM final concentration in culture media.

Kanamycin		
Reagent	Final concentration	Amount
Kanamycin	50 mg/mL	0.5 g
ddH_2O	n/a	10 mL
	Total:	**10 mL**

Note: Prepare stock solution at 50 mg/mL, sterilize it using a 0.22 μm filter, store it at −20°C, and use it at the appropriate final concentration in growth media.

⚠ CRITICAL: Kanamycin causes allergic respiratory and skin reactions; it may also cause difficulty breathing and damage fertility or a fetus. Wear protective gloves and avoid breathing dust.

Apramycin		
Reagent	Final concentration	Amount
Apramycin	50 mg/mL	0.5 g
ddH_2O	n/a	10 mL
	Total:	10 mL

Note: Prepare stock solution at 50 mg/mL, sterilize it using a 0.22 μm filter, store it at −20°C, and use it at the appropriate final concentration in growth media.

⚠ CRITICAL: Apramycin may irritate skin or eyes; it may also cause difficulty breathing and damage to fertility and fetuses. Wear protective gloves and avoid breathing dust.

Alternatives: Teknova supplies 50 and 200 μg/mL Km LB plates, as well as 50 and 100 μg/mL Apr LB plates.

STEP-BY-STEP METHOD DETAILS

Integrating CRAGE and CRAGE-Duet LPs

Timing: 5–10 days (user-dependent)

This section describes step-by-step methods for conjugal transformation of pW17 and pW37, as well as integration of the CRAGE and CRAGE-Duet LPs into the genomes of recipient bacteria. The step numbers correspond to those of the Graphical Abstract and Figure 1.

1. Preparation of donor and recipient strains.
   a. Culture donor strain BW29427 harboring pW17 or pW37 LP plasmid in LB medium supplemented with 0.3 mM diaminopimelic acid (DAP) and 50 μg/mL Km at 37°C in an incubation shaker at 200 rpm overnight.
   b. Culture the recipient bacteria using their optimal growth conditions.

Note: With improper handling, bacteria can contaminate media, and cross-contamination can occur between cultures. Use proper sterilization techniques. If working in a laminar flow hood, make sure not to block airflow inside it. Use filter tips and change them often.

Always include a blank control culture with the media being used. Follow necessary practices to prevent contamination.

2. Conjugal transformation of the LP plasmid.
   a. Measure optical density at 600 nm (OD_{600}) for the donor and recipient cultures and mix with a 4:1 donor to recipient ratio in a 2 mL tube (see notes below). Centrifuge this mixture (18,000 × *g*, 1 min) and wash three times with 1 mL LB to remove residual antibiotics from the culture.

Note: For landing pad integration, we typically mix 400 μL to 1 mL of OD=2 donor strain with 100–250 μL of OD=2 recipient strains. Users can increase conjugation efficiency by changing the donor to recipient ratio in either direction. Some recipient strains can be conjugated in less than 6 h, while other strains may take more than 24 h. For strains with lower conjugation efficiency, consider increasing donor and/or recipient strains by 2- to 5-fold as well as increasing conjugation incubation time.

   b. Resuspend the resulting pellet in 50 μL LB with 0.3 mM DAP media and transfer onto a nitrocellulose filter membrane on top of an LB agar plate containing 0.3 mM DAP (Figure 2A).

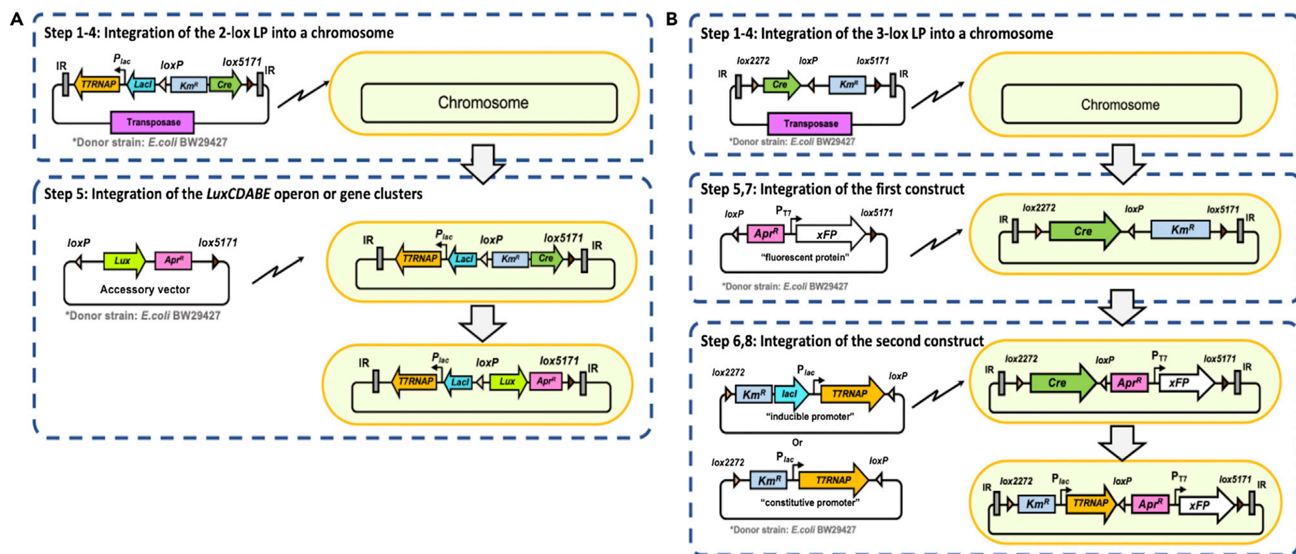

Figure 1. Schematic diagram of the CRAGE and CRAGE-Duet systems

(A) The CRAGE system. A landing pad (LP) transposon (containing *Cre* and Km^R, flanked by *loxP* and *lox5171*) on a pW17 plasmid is integrated into the genomes of recipient strains (steps 1–4). The payloads are then integrated into the LP site via Cre-mediated cassette exchange (step 5).

(B) The CRAGE-Duet system. An LP transposon (containing *Cre* flanked by lox2272 and loxP and Km^R flanked by loxP and lox5171) on a pW37 plasmid is integrated into the genomes of recipient strains (steps 1–4). The payloads are subsequently integrated into the first and second sites of the LP via Cre-mediated cassette exchange (steps 6–8).

Incubate this plate at 28°C (or at an optimal temperature for the target strain) for 12–24 h (Figure 2B).

Note: Use strain-specific temperatures for conjugation. We used 28°C because it was the optimal temperature for many bacteria with which we worked.

Researchers can use LB with 0.3 mM DAP instead of LB throughout steps 2a–2c to simplify the procedure.

Include a blank control to confirm that the procedure is contamination-free. To do this, pipette 25 μL LB and/or LB with DAP that was used for wash and resuspension steps (steps 2a–2c) onto an empty LB with DAP agar plate and incubate with other experimental samples.

Although multiple conjugations can be done on the same plate, we strongly recommend using separate or multi-well plates to prevent contamination (Figure 2C).

c. Scrape off the bacterial mixture grown on the membrane using an inoculation loop and resuspend it into 500 μL LB media. Then centrifuge it (18,000 × *g*, 1 min), wash it with 1 mL LB three times, and resuspend it in 1 mL LB. Plate 100 μL of the mixture on an LB plate containing the appropriate concentration of Km.

Note: Single colonies can also be streaked out simply on the selection plate using an inoculation loop.

Depending on the conjugation efficiency, users might consider plating the mixture with 10^{-1}, 10^{-2}, or 10^{-4}-fold dilution onto selection plates.

Although freezing will compromise conjugation efficiency, users can freeze the conjugation mixture and store it by adding glycerol to a final concentration of 15%.

d. Incubate the plate at an optimal temperature for the growth of the recipient bacteria until single colonies appear (Figure 2D, 1–3 days, strain-dependent); then restreak single colonies on selection media.

3. Screening and verifying LP integration.
   a. Inoculate single colonies onto 96-well plates containing 150 μL of LB media with 7.5% glycerol and an appropriate concentration of Km, determined using the MIC test for the recipient strain.
   b. Use 0.5 μL of each overnight culture in a 20 μL PCR reaction to determine whether the LP has integrated into the genome or has remained as a plasmid (Figure 3).
      i. For the CRAGE system, primers SBP572 (CCACCTTCGTAAGACTGTAGTG) and 99W (TCCCAGATCTCAAACTGGAACAACACTC) amplify a 374 bp LP region, and primers SBP202 (GAAAAGCTGGGCGCGTTAAGCCAGCCCCGACACCCG) and SBP203 (GGGGC TGGCTTAACGCGCCCAGCTTTTCAATTC) amplify a 645 bp plasmid backbone region.
      ii. For the CRAGE-Duet system, primers 197_DT (GCTATCAGGACATAGCGTTGGCTACC) and 190_DT (GTAATGCTCTGCCAGTGTCGCG) amplify a 423 bp LP region, and primers 197_DT (GCTATCAGGACATAGCGTTGGCTACC) and 44W (CGCTGCATAACCCTGCTTC GG) amplify a 859 bp backbone region. If the LPs are integrated into the genome of the recipient bacteria, only the LP region is amplified.

PCR reactions mixture

Reagents	Final concentration	μL/reaction
Bacteria template	n/a	0.5
5 M betaine	0.5 M	2
Forward primer (10 μM)	0.2 μM	0.4
Reverse primer (10 μM)	0.2 μM	0.4
HiFi 2× MasterMix	1×	10
ddH_2O	n/a	6.7
	Total:	20

PCR reactions cycle

Steps	Temperature (°C)	Time	Cycles
Initial denaturation	98	30 s	1
Denaturation	98	5 s	30–35
Annealing	60	10 s	
Extension	72	30 s–4 min	
Final extension	72	2–5 min	1
Hold	4	Forever	

   c. To identify the integration location of the LP, isolate genomic DNA from 2 mL overnight culture using QIAGEN blood and tissue kit followed by Illumina or PacBio sequencing, with 10–500 ng or 2–5 μg input DNA, respectively. Users can map the raw reads to the LP using Geneious Prime or Integrative Genomics Viewer (IGV). This allows the user to identify the reads that map both 3′- and 5′-ends of the LP as well as the LP integration site. Users can then map the flanking sequences that correspond to the genome to identify the integration location. Alternatively, users can use inverse PCR (Ochman et al., 1988) at the junctions of LP and genomic DNA combined with Sanger sequencing to determine the LP integration site (Liu et al., 2020).

Note: Typically, we pick 8–16 colonies from each conjugal transformation plate.

Some of the conjugation steps (e.g., mixing and washing) are contamination prone; therefore, we recommend using 16S rRNA sequencing to ensure the integrity of the strains.
The amount of input DNA for sequencing may vary depending on the requirement of each sequencing facility. Follow the guidelines from each facility.

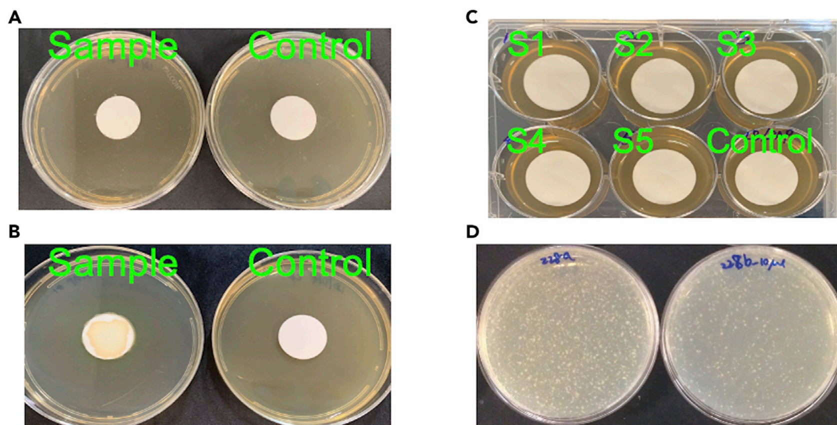

Figure 2. Conjugation on LB/DAP agar plates

(A) A conjugation mixture was spotted on nitrocellulose filter membranes placed on top of LB/DAP agar plates. LB/DAP medium was used as a negative control to ensure that no contamination occurred during the conjugation procedure.

(B) Conjugation plates after 24 h incubation.

(C) Multi-well plates were used to increase conjugation throughput.

(D) Single colonies from the conjugation mixture appeared on the LB plate containing Km. Different dilutions were plated.

4. Testing the growth phenotype to evaluate the impact of the LP integration.
   a. Culture the LP-integrated strains and their corresponding wild-type strain in 1 mL LB with appropriate antibiotics at the strain-specific optimal growth temperature to reach an OD_{600} of 2–6.
   b. Centrifuge cells (18,000 × *g*, 1 min), wash them with M9 minimal media three times and resuspend them into a fresh medium. Dilute this culture to an OD_{600} of 0.1.
   c. Add 150 μL of cultures to each well of a 96-well black/clear flat-bottom plate (Corning). Perform the growth assay in triplicate for each strain.
   d. Use a Synergy H1 microplate reader (BioTek) to measure the growth of the LP strains at intervals of 15 min for 20–48 h at a strain-specific optimal growth temperature with constant shaking (Figure 4).
   e. Select the LP strains with growth phenotypes more similar to those of the wild-type strain for subsequent study. Information about the LP insertion location identified in the previous section also supports the selection of the target strain.

Note: In our experience, almost all strains grow well. However, we sometimes see obvious growth differences between WT and LP strains. Strains whose growth is not compromised will be carried over to the downstream experiment.

Integrating the payload into the LPs

Timing: 5–10 days (user-dependent)

5. Integrating a *lux* operon to confirm LP function.
   a. Conjugally transform pW34 containing the *luxCDABE* operon into the LP strain. Because of the high conjugation and integration efficiency, we recommend streaking out a single colony directly from the conjugation mixture (Figure 5A).
   b. Screen 8 to 10 single colonies from each conjugation for their resistance to Apr and sensitivity to Km through counterselection (Figure 5B).
   c. Inoculate the positive integrants of the *luxCDABE* operon in 1 mL of LB medium with appropriate antibiotics and grow them at optimal temperature overnight.

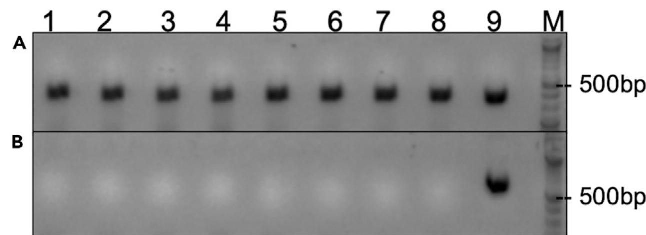

Figure 3. CRAGE landing pad integration screening

(A and B) Agarose gel electrophoresis (1% agarose) of PCR products using (A) the CRAGE LP or (B) backbone-specific PCR primer sets. Lanes 1–8 were from LP integrants. Lane 9 was from pW17 conjugation plasmid DNA used as a template. Lane M is an NEB 2-log ladder.

d. Dilute these cultures to an OD_{600} of 0.1 with fresh LB medium containing appropriate antibiotics.
e. Transfer 4 × 150 μL aliquots of each culture to a 96-well plate with a clear bottom. Add IPTG to these cultures at final concentrations of 0, 0.01, 0.1, and 1.0 mM. Incubate the cultures in the Synergy H1 microplate reader at the optimal growth temperature for 24 h. Measure the OD and bioluminescence at intervals of 15 min (Figures 5C and 5D).

Note: Once successful integration of the *luxCDABE* operon and luminescence activity is confirmed, the LP strain is ready for the downstream payloads integration steps.

Single constructs or libraries of constructs can be transformed and directly integrated into the set of both CRAGE and CRAGE-Duet LP strains. Multi-well plates can help improve throughput.

Because the transposon is randomly integrated into the genomes, the LP may be accidentally integrated into a location essential to the growth of the recipient strains. In our experience, an optimal LP strain, in which the LP is inserted in the non-polycistronic intergenic region, is usually found if 10 transconjugants are selected at step 2. In addition to identifying the LP integration location and evaluating the growth phenotype, we recommend that users perform assays for other phenotypes that are important in downstream scientific applications.

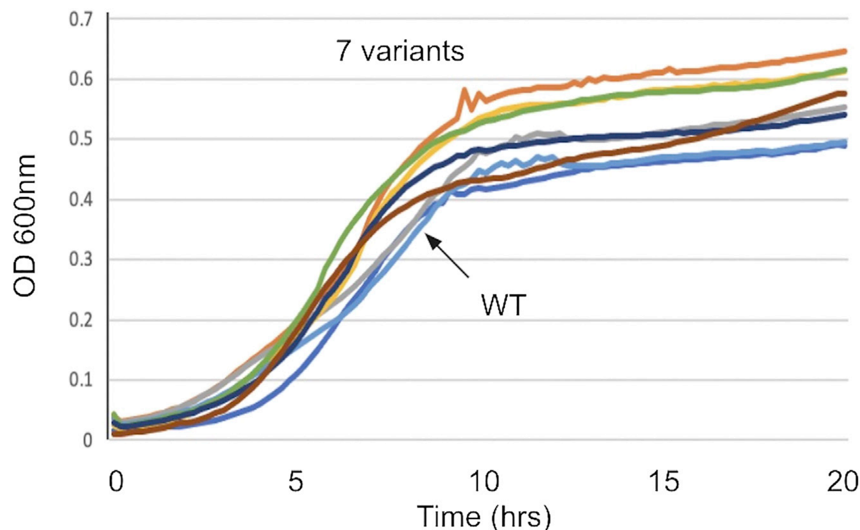

Figure 4. Growth curve comparison between wild-type and CRAGE LP-integrated strains

One wild-type (WT) *Serratia odorifera* and its seven CRAGE variants were incubated in a Synergy H1 microplate reader with shaking for 20 h at 28°C. The optical density (OD) was measured at intervals of 15 min. No obvious differences were seen between the CRAGE LP strain and its WT.

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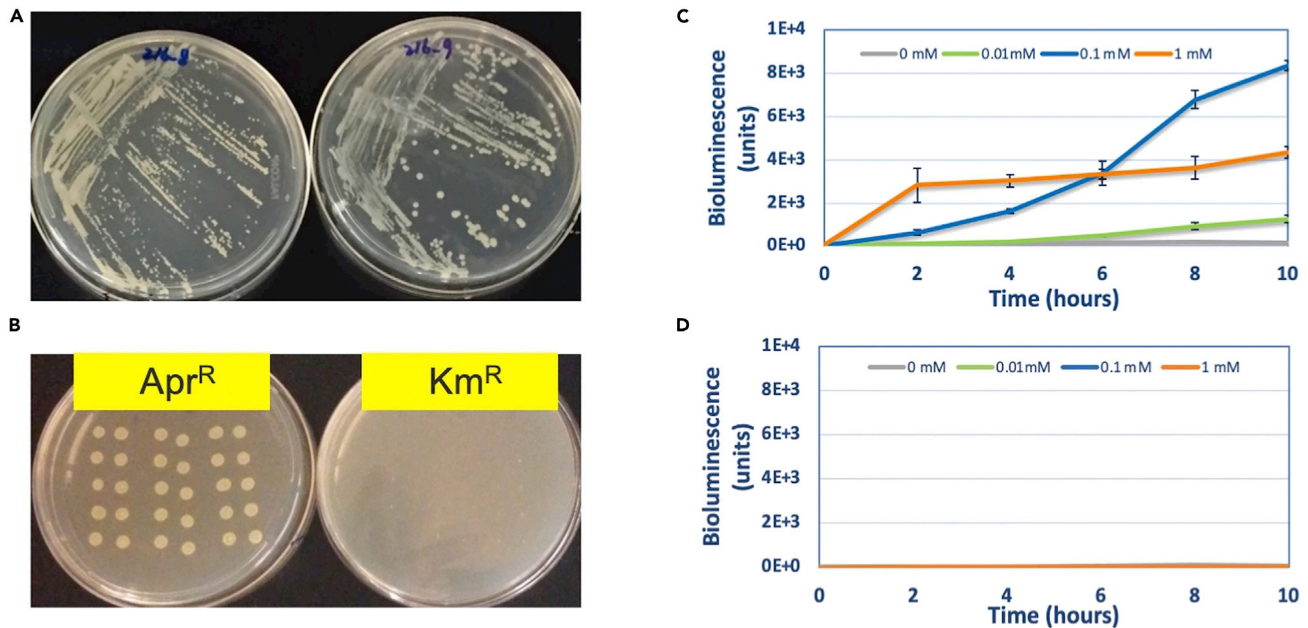

Figure 5. Integration of the *luxCDABE* operon and luminescence assay of the integrated strains

(A) The second-step conjugation mixture was directly streaked out on the selection media.

(B) 1 μL of each of the overnight cultures was spotted on both Apr and Km plates at appropriate concentrations. Strains gained Apr resistance and lost Km, indicating successful cassette exchange of the Km resistance gene with the Apr resistance gene and the *luxCDABE* operon.

(C and D) The *Pseudomonas simiae* strain with the *LuxCDABE* operon integrated (C) and its corresponding LP strain (D) were induced under different amounts of IPTG and incubated in the Synergy H1 microplate reader with shaking for 10 h at 28°C. Bioluminescence was measured at intervals of 15 min. Error bars from three different biological replicates are shown for the *LuxCDABE*-operon-integrated strain (C). No obvious bioluminescence was detected in the LP-only strain (single replicate). Bioluminescence increases at a higher rate with increased IPTG.

Integrating the second payload into the LPs (CRAGE-Duet only)

Timing: 5–10 days (user-dependent)

6. Integrating the inducible or constitutive promoter.

For the CRAGE-Duet system, the second payloads are delivered to the second integration site. As a proof-of-principle experiment, use donor strains with T7RP under IPTG-inducible promoter pW38 and/or constitutive promoter pW39 for the conjugation at the second integration site (lox2272 and loxP). Select strains in which T7RP has been integrated by their resistance to both Km and Apr (indicating successful cassette exchange), followed by a luminescence assay (refer to step 5 for details).

Implementing the CRAGE-CRISPR system

Timing: 5–10 days (user–dependent)

Users can use 3-lox LP strains created from steps 2 and 3 (described above) for all the downstream conjugation and verification steps.

7. Once the LP for the CRAGE-Duet system is successfully integrated into the genome, implement the CRAGE-CRISPR system in these strains. Use the plasmids pR6K-2L-SpCas9 and pR6K-2L-dCas9-RNAPω to implement CRISPRi and CRISPRa, respectively. These plasmids are targeted

to the first integration site between loxP and lox5171. Follow the step 5 protocol to integrate the payloads on these plasmids into the LP site.

8. Use pR6K-lox2272WT as a base plasmid to integrate sgRNA constructs for the CRISPR systems. Modify these plasmids to target sequences of interest. Follow the step 6 protocol to integrate the payloads into the second integration site of the CRAGE-Duet LP.

Once the LP is integrated into the genome, integrating payloads using the *Cre*-mediated cassette exchange is generally more efficient than mariner-transposase-mediated transposon integration. Although we generally use a 1 mL culture of recipient strain, 100 μL is often sufficient for conjugal integration of payloads into the recipient LP strain. If the efficiency of the conjugal recipient strain is very high, users can shorten the conjugation incubation time to optimize the protocol.

EXPECTED OUTCOMES

The main outcome of this protocol is the rapid integration of simple to large complex payloads into non-model bacteria. Successful implementation of CRAGE and/or CRAGE-Duet allows functional genomics studies of non-model bacteria, engineering of members of microbial communities, and acceleration of the Design–Build-Test–Learn (DBTL) cycle (Liu et al., 2015) for industrial strain development. All plasmids described in the protocol are available to the scientific community upon request or purchased through Addgene.

LIMITATIONS

We have demonstrated that the CRAGE technology can be used to engineer more than 40 species including α-, β- and γ-*Proteobacteria* and some *Actinobacteria* (Wang et al., 2019). We are currently expanding this portfolio. We have tested payload constructs ranging from < 1 kbp to 60 kbp. The integration efficiency remained constant regardless of payload size. The integration limit likely depends on the size of the DNA fragment the BAC-based accessory plasmid can carry, which is about 300 kbp.

TROUBLESHOOTING

Problem 1

Conjugation tends to introduce cross-contamination.

Potential solution

At any step in which multiple recipient strains are handled in parallel, cross-contamination tends to occur. Even minor contamination in one strain can cause cross-contamination in strains that have high conjugation efficiency ($1–10^{10}$ colony-forming units/conjugation). Because conjugation efficiency changes dramatically depending on the strain, perform every procedure with extra caution to prevent any contamination. Always include blank culture media as controls in all culture and conjugation steps. We also highly recommend checking the identity of strains using 16S rRNA sequencing to confirm the correct strain at each critical step.

Problem 2

LP colony PCR not working.

Potential solution

Do not allow the volume of overnight culture used in the colony PCR to exceed 2.5% of total PCR volume (0.5 μL cells in 20 μL PCR reaction). Depending on the strain and the cell density of its culture, we found that diluting the overnight culture by 20- to 40-fold facilitates the colony PCR. Betaine is a well-known PCR additive that helps reduce the formation of secondary structures caused by GC-rich regions. 0.5 mM betaine is the best solution for all PCRs in our experience. The addition of betaine also helps the PCR amplification of genomes with high GC content.

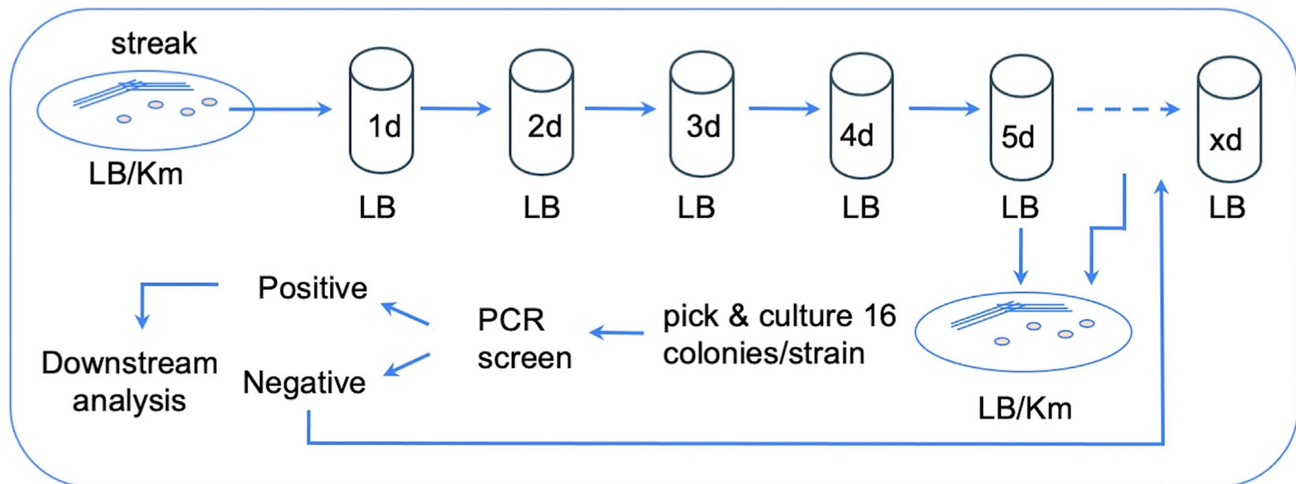

Figure 6. Plasmid curation through serial sub-culturing

Cells is streaked on LB plates with an appropriate concentration of antibiotics. A single colony is picked and passes several generations (with 200- to 500 -fold dilution) on LB media without selective pressure. Cultures are restreaked on a selection plate periodically and screened with PCR. The positive clones are picked, followed by the downstream analysis of 16S confirmation, LP location detection, and function test.

Problem 3

The LP transposon does not jump into the genome.

Potential solution

If pW17 and pW37 can replicate in the recipient strains (which is sometimes the case in species that are genetically closer to *E. coli*), the LP is sometimes not integrated into the genome, resulting in no positive clones at step 3. We cure the plasmid as the solution to this problem. To do this, we pass transformants serially in fresh LB medium without antibiotics for 10–30 days. We streak out the culture on LB containing appropriate antibiotics periodically for PCR screening to identify the successful integrants, as outlined in Figure 6. The curation period is greatly strain-dependent.

Problem 4

The LP is rearranged upon transposition into the genome.

Potential solution

We recommend sequencing the whole landing pad fragment to make sure that this region was inserted accurately. We often found that species of β-*Proteobacteria* are troublesome upon transposition. The lox site might be missing, or the LP cassette might get rearranged. For some β-*Proteobacteria* strains, we have overcome this problem by using a minimal CRAGE vector without *Cre* and then transiently expressing it using a delivery vector for the downstream cassette exchange steps. We will make the modified vector available to the scientific community upon request.

Problem 5

Payloads are not integrated into the genome.

Potential solution

When using pW5Y-based accessory vectors, the constructs tend to remain in the plasmid format in the bacteria, especially for species of *Enterobacteriaceae* (Figure 7). In this case, use simple curation procedures similar to the problem 3 solution (which explains how to cure the plasmid) to screen the strains in which the cassette exchange was successful (those strains will be resistant to Apr and susceptible to Km).

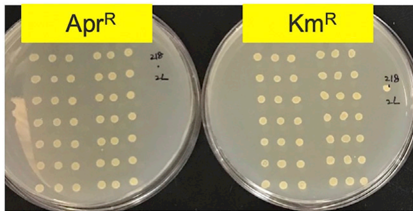

Figure 7. Example of failed cassette exchange

The image shows single colonies picked from step 5 that did not pass counterselection; the strains were resistant to both Apr and Km. This may indicate that *Cre*-lox recombination did not occur and that the accessory vector remained in the plasmid form in recipient strains.

We also recommend using pW34, which is based on the R6Kγ origin of replication and cannot replicate in recipient microbes.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Yasuo Yoshikuni (yyoshikuni@lbl.gov).

Materials availability

Some plasmids generated in this study have been deposited to Addgene: pW17 (Cat# 158207), pW34 (Cat# 158210), and pW5Y (Cat# 158211). Other plasmids and strains can be requested from the lead contact upon completing a Materials Transfer Agreement.

Data and code availability

The published article includes all datasets generated or analyzed during this study.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

Z.Z., Y.Y., and J.F.C. designed the study; Z.Z. performed the experiments; Z.Z. and Y.Y. wrote, reviewed, and approved the manuscript. J.F.C. reviewed and approved the manuscript.

DECLARATION OF INTERESTS

Lawrence Berkeley National Laboratory filed a United States patent application for CRAGE technology (US patent 20190048354). The application lists Y.Y., G.W., Z. Zhao., J.F.C., and D.R. as inventors.

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