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# CRISPR–Cas9, CRISPRi and CRISPR-BEST-mediated genetic manipulation in streptomycetes

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**Streptomycetes are prominent sources of bioactive natural products, but metabolic engineering of the natural products of these organisms is greatly hindered by relatively inefficient genetic manipulation approaches. New advances in genome editing techniques, particularly CRISPR-based tools, have revolutionized genetic manipulation of many organisms, including actinomycetes. We have developed a comprehensive CRISPR toolkit that includes several variations of 'classic' CRISPR–Cas9 systems, along with CRISPRi and CRISPR-base editing systems (CRISPR-BEST) for streptomycetes. Here, we provide step-by-step protocols for designing and constructing the CRISPR plasmids, transferring these plasmids to the target streptomycetes, and identifying correctly edited clones. Our CRISPR toolkit can be used to generate random-sized deletion libraries, introduce small indels, generate in-frame deletions of specific target genes, reversibly suppress gene transcription, and substitute single base pairs in streptomycete genomes. Furthermore, the toolkit includes a Csy4-based multiplexing option to introduce multiple edits in a single experiment. The toolkit can be easily extended to other actinomycetes. With our protocol, it takes <10 d to inactivate a target gene, which is much faster than alternative protocols.**

## Introduction

Bacteria of the genus *Streptomyces* and their relatives have served as the most promising source of antimicrobials for >70 years. They provide >70% of currently used antibiotics[1,2]. However, the current natural products–based antibiotics discovery and development pipeline is suffering from diminishing returns. No new classes of antibiotics have been developed over the recent decades[3], which heightens the global health threat of multidrug-resistant infectious diseases.

Fortunately, modern genome mining[4] reveals that the genomes of streptomycetes and other related actinomycetes still possess a large unexploited potential to encode novel natural products[5]. To unlock this potential genetically, advanced technologies to efficiently manipulate and engineer the genomes of the producer strains are required[5,6]. However, it is more difficult to edit their genomes than those of other unicellular model microorganisms such as *Escherichia coli* and *Saccharomyces cerevisiae*, because of their mycelial growth, intrinsic genetic instability, and GC-rich (>70%) genome. Moreover, only a few genetic manipulation methods are available for actinomycetes.

The recent availability of clustered regularly interspaced short palindromic repeats (CRISPR)–CRISPR-associated (Cas)-based genome editing technologies has revolutionized the biotechnology field. CRISPR–Cas-mediated genetic manipulation approaches have been successfully applied to organisms across all domains of life[7,8] relatively quickly and efficiently, with no scarring.

To develop advanced genetic manipulation methods for exploiting new bioactive natural products from streptomycetes, we first developed a toolkit using classic DNA double-strand break (DSB)-based CRISPR–Cas9 and its relatives[9]. Along with similar tools established by others[10–12], CRISPR–Cas9 markedly increased the ease of genetically manipulating streptomycetes[9,13,14]. However, concerns about DSB-mediated genome instability and Cas9-related toxicity remained[13,14]. Accordingly, we then established a CRISPR base editing system, CRISPR-BEST[15], which provides editing efficiencies

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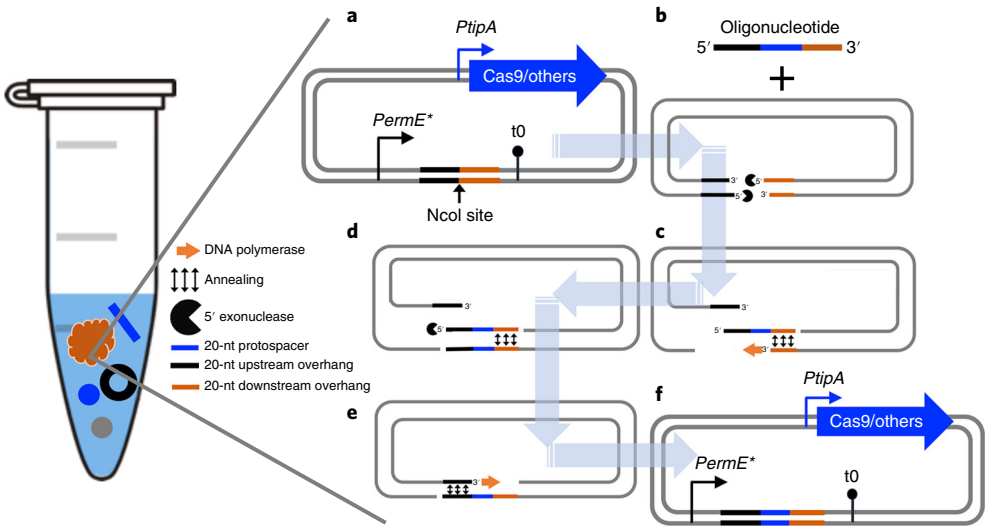


**Fig. 1 | A mechanistic overview of the all-in-one pot ssDNA bridging method for sgRNA cloning. a**. Linearize the plasmid with NcoI. **b**–**f**, Illustration of ssDNA bridging. **b**, The 5′ exonuclease included in the NEBuilder mix digests the 5′ ends of the double-stranded DNA (dsDNA), making the 3′ complementary ends available. **c**, The nondigested ssDNA oligonucleotide anneals to the exposed 3′ end of the plasmid backbone; the DNA polymerase extends the 3′ end, using the oligonucleotide as the template. **d**, The 5′ exonuclease then removes the original protospacer containing the ssDNA oligonucleotide. **e**, The other nicked strand of the plasmid anneals to the newly synthesized 3′ end of the complementary strand and then is extended by the DNA polymerase. **f**, The DNA ligase included in the NEBuilder mix then seals the nicks to form the desired protospacer-cloned CRISPR plasmid. *PermE**, a widely used constitutive promoter; *PtipA*, thiostrepton-inducible promoter; t0, terminator. Blue arrows represent one of the key components of CRISPR plasmids; 'others' represents dCas9 or Cas9n–deaminase.

comparable to that of the classic CRISPR–Cas9 systems without introducing DSBs—and with editing resolutions reaching the single-nucleotide level[9,15,16]. To further simplify the single-guide RNA (sgRNA) cloning step, we established an efficient single-strand DNA (ssDNA) oligonucleotide bridging method (Fig. 1) and optimized the *E. coli–Streptomyces* conjugation protocol. To meet the increasing demands for introducing multiple mutations in a single experiment, we developed a multiplexing option[15], which uses the Csy4 (also known as type I-F CRISPR-associated endoribonuclease Cas6f[17])-based sgRNA self-processing machinery. Together with the protospacer finder CRISPy-web[18,19], the CRISPR–Cas9 based toolkit[9] and the CRISPR-BEST toolkit[15] provide a versatile genetic manipulation system for streptomycetes (Fig. 2).

## Comparison with other currently available genetic manipulation methods

Traditional mutagenesis methods were established in 1978, when for the first time external DNA was successfully transferred into streptomycetes[20]. For example, plasmids with temperature-sensitive and unstable replicons were used for classic double-crossover–based mutagenesis[21], which relies on naturally occurring homologous recombination events. Later, PCR-targeting[22] became popular; this method relies on identifying the editing target in a cosmid/fosmid library, followed by a λ-Red-mediated gene disruption or deletion of the target encoded on the cosmid/fosmid in *E. coli*, introducing the modified cosmid/fosmid into the native producer strain and, finally, screening for double-crossover events[21,22]. To recycle the resistance marker, it can be removed by Cre/lox, although that leaves an 81-bp scar[22]. However, this method is relatively intricate, because it requires a fully characterized genomic library of all target strains.

Besides the PCR-targeting method, a *S. cerevisiae* meganuclease *I-SceI*-based gene deletion method for streptomycetes was established in 2014[23]. The use of this method requires two conjugations: the first integrates the 18-bp *I-SceI* recognition site together with the homologous recombination templates (editing templates) into the genome of the target strain by a single crossover event. In a second conjugation, the meganuclease *I-SceI* is introduced; upon expression, it cuts the 18-bp *I-SceI* recognition site, leading to a DSB in the chromosome. Subsequently, homologous recombination-based DSB repair leads to either the desired deletion without a scar or to the wild-type (WT) genotype. Owing to the selection pressure introduced by the DSB, the editing (double crossover)

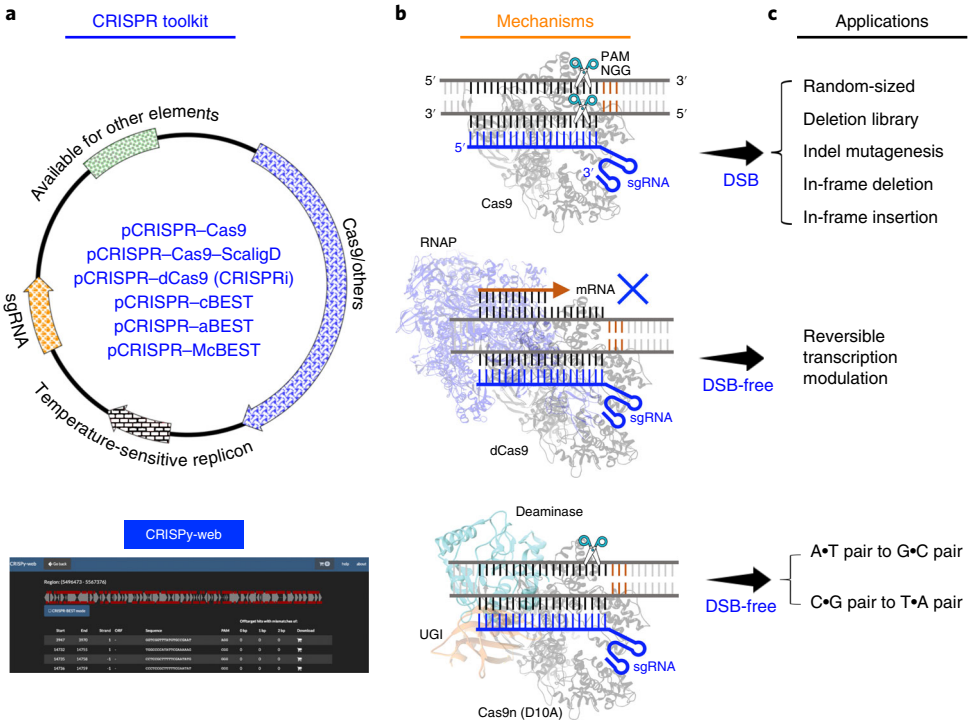


**Fig. 2 | An overview of the CRISPR-based genetic manipulation system included in this protocol. a**, The components of the CRISPR-based streptomycetes/actinomycetes genetic manipulation toolkit. The toolkit includes six plasmids (listed inside the plasmid map) and an sgRNA design tool, CRISPy-web. 'Available for other elements' means that there is space in this plasmid system that can be used for cloning additional elements, for example, the editing templates or Csy4. 'Cas9/others': represents the Cas9 component of the CRISPR plasmids; 'others' can include dCas9, Cas9n-adenosine deaminase, or UGI-Cas9n-cytidine deaminase; this part varies with the different plasmids in the toolkit. **b**, A simple mechanism of action for each type of Cas9 used in the CRISPR system is provided. pCRISPR-Cas9 and pCRISPR-Cas9-ScaligD: sgRNA binds to Cas9, forming a Cas9-sgRNA complex, and then guides the complex to find and bind to the target DNA region; then Cas9 cleaves the target DNA to introduce a DSB, and the DSB can be repaired by different ways, leading to the genome-editing events. pCRISPR-dCas9 (CRISPRi): The dCas9-sgRNA complex preserves DNA binding ability but has no DNA cleavage activity. It therefore blocks the transcription process mediated by the RNA polymerase when guided to the target sequence by the sgRNA. pCRISPR-aBEST, pCRISPR-cBEST, and pCRISPR-McBEST: A deaminase is fused to Cas9n, and then the whole complex is guided by sgRNA to the target DNA region, where adenosine deaminase can convert A●T pairs to G●C pairs, and cytidine deaminase can convert C●G pairs to T●A pairs, which are shown in **c**. **c**, The applications of different CRISPR tools within the CRISPR-based genetic manipulation system are summarized. PAM, protospacer-adjacent motif (in this protocol, all CRISPR plasmids use the same PAM, 5′-NGG-3′); RNAP, RNA polymerase; UGI, uracil glycosylase inhibitor.

efficiency of this method is higher than that of the PCR-targeting method. However, the protocol for this meganuclease *I-SceI*-based method is still relatively difficult and time consuming.

## Development of the protocol

Tong et al.[16] described the initial protocol for using our CRISPR–Cas9-based toolkit. With years of practical use and continual extension of the toolkit, we have updated the protocol for better performance and will described it here. However, some concerns and challenges remain with this technology[13,14], including, for example, the problem that overexpression of active Cas9 is toxic in many streptomycetes and may lead to unwanted off-target effects[13,14]. Moreover, most streptomycetes have a linear chromosome that is relatively unstable and susceptible to large-scale chromosomal deletions and rearrangements[24]. DSBs in the terminal inverted repeats of the chromosomes are speculated to be the main triggers of genome instability[15,25]. To address the above-mentioned concerns, we additionally developed a highly efficient, DSB-free, precise, and multiplex genome editing toolkit for streptomycetes, CRISPR-BEST[15], which is based on deaminase-mediated base editing technology[26–28].

Given the complexity of intracellular metabolic networks, it is often necessary to engineer multiple genes simultaneously for both basic and applied studies. With classic actinomycete genetic

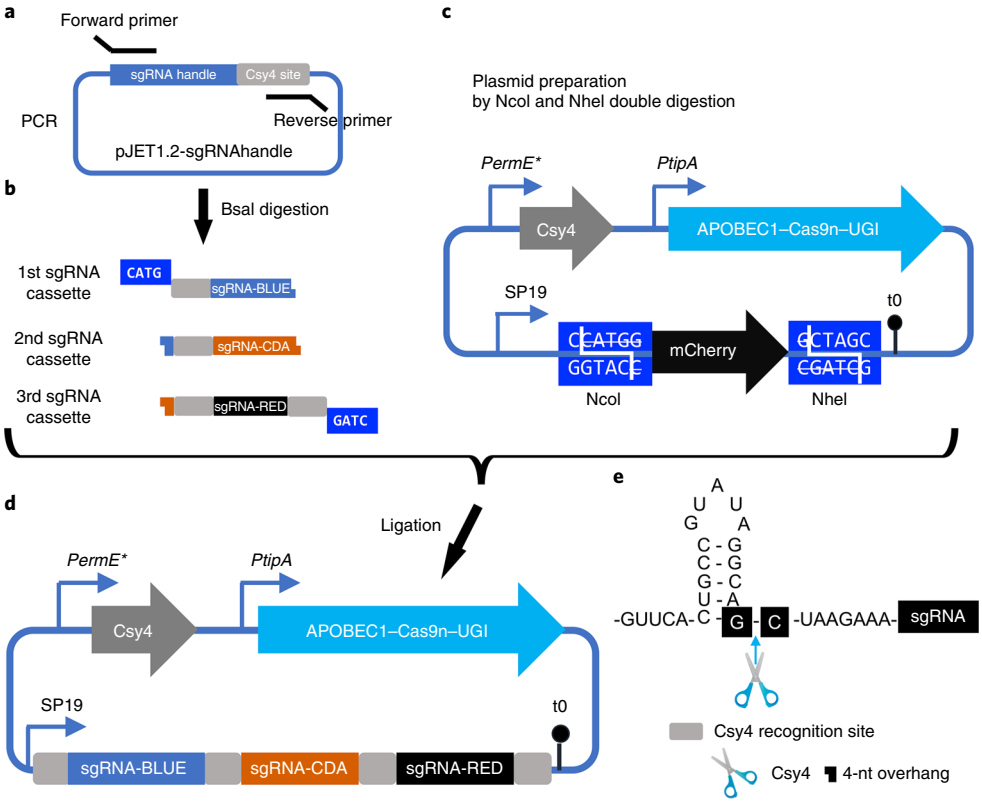


**Fig. 3 | A three-sgRNA multiplexed example. a**, PCR-amplify all sgRNA fragments; for detailed sgRNA design[15], refer to Step 8B(i) and Box 1. **b**–**d**, An overview of the two-step Golden Gate assembly. **b**, Preparation of the sgRNA fragments by BsaI digestion. **c**, Preparation of the plasmid backbone by NcoI and NheI digestion. **d**, Ligation of the prepared fragments from **b** and **c** to obtain the final, ready-to-use CRISPR plasmid. **e**, Illustration of the mechanism of action of Csy4-based RNA processing. SP19, synthetic promoter19 (ref. [51]); Csy4 site, Csy4 recognition and cleavage site; sgRNA-BLUE, the sgRNA targets the *SCO5087* gene of the actinorhodin biosynthetic gene cluster; sgRNA-CDA, the sgRNA targets the *SCO3230* gene of the calcium-dependent antibiotic (CDA) biosynthetic gene cluster, sgRNA-RED: the sgRNA targets the *SCO5892* gene of the undecylprodigiosin (RED) biosynthetic gene cluster.

engineering methods, only a single genetic modification can be introduced per round of genetic manipulation. However, owing to its simplicity and modularity, CRISPR inherently provides the capability for multiplexing. The current multiplexing applications in streptomycetes are still limited by the efficacy of individual sgRNA generation. This requires an independent promoter and terminator for each individual sgRNA transcription unit, increasing the risks of plasmid instability and uneven distribution of each sgRNA, along with the potential of triggering unwanted mutagenesis[29–31]. To address the aforementioned concerns, we therefore designed a Golden Gate assembly–compatible Csy4-based[17] sgRNA self-processing system that requires only a single promoter and terminator pair for multiple sgRNAs separated by the Csy4 recognition sites. A simple example of a three-sgRNA multiplexed plasmid design and construct is shown in Fig. 3.

## Overview of the procedure

Figure 4 provides a summary of all approaches. The overall procedure can be divided into four (optionally, five) main stages, once the goal (e.g., inactivation of a gene of interest) is chosen: stage I—in silico design of sgRNA, primers, and (optional) editing templates (Steps 1–7, 8C(i); stage II—construction and validation of the designed CRISPR plasmid (Steps 8–20); stage III—conjugation of the correctly constructed CRISPR plasmid to the target streptomycetes (Steps 21–33); stage IV—validation of the correctly edited target streptomycetes (Step 34); and (optional) stage V—removal of the CRISPR plasmids (in the following, this is referred to as 'curing') from the correctly edited streptomycete strains to make a clean, CRISPR-plasmid-free mutant that is ready for a second round of genetic manipulation (Steps 35–39).

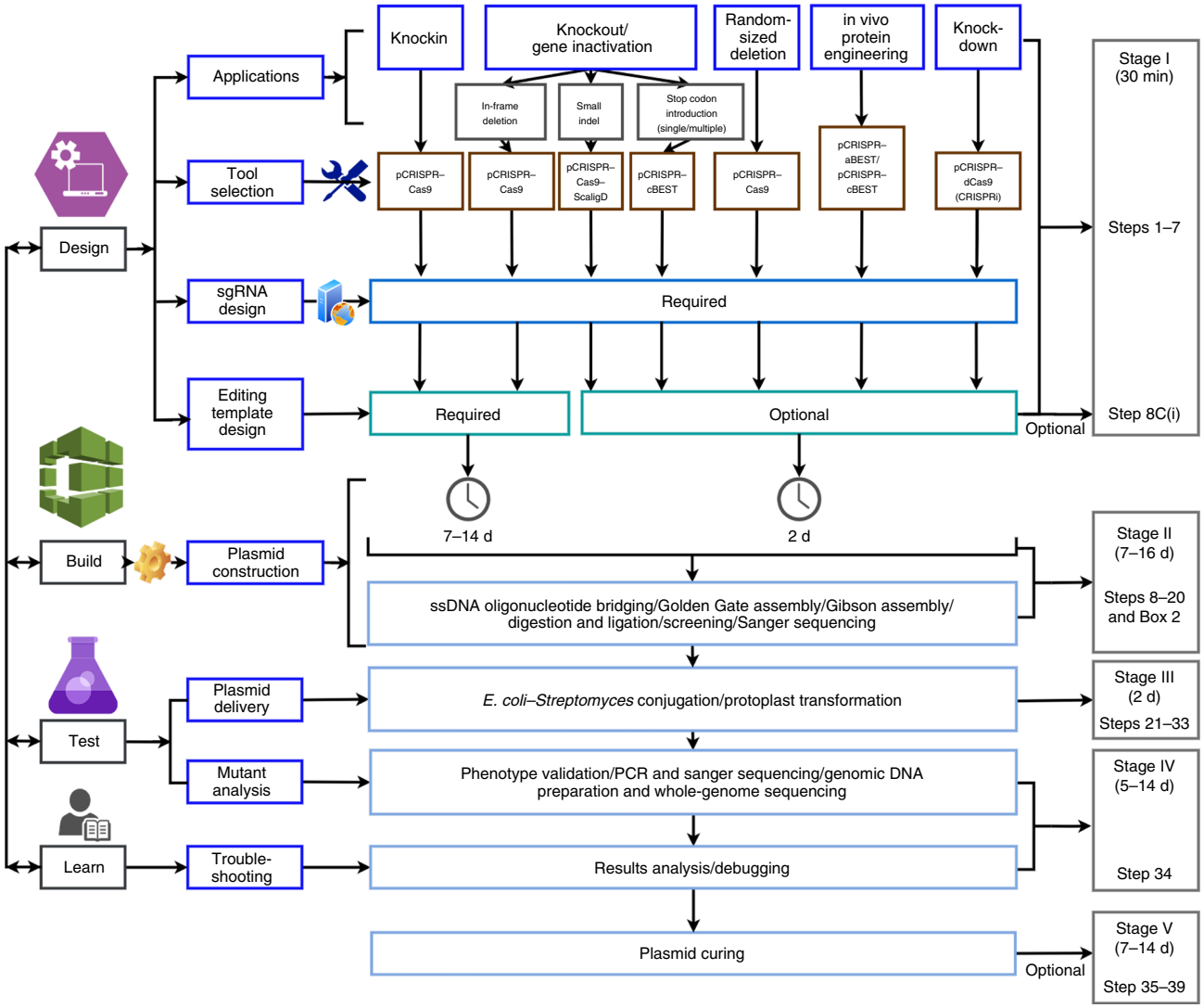


**Fig. 4 | An overview of the entire protocol.** Shown is a schematic representation of processes for using different CRISPR plasmids within this protocol to complete desired genetic manipulation in the target streptomycete strain.

## Applications of the method

Primarily, we demonstrated the use of the CRISPR toolkit presented in this protocol in *S. coelicolor* and *S. collinus*[9,15], but over time it has also been applied in many other streptomycetes, such as *S. albus*[32], *Streptomyces sp.* SD85[33], and a series of environmental *Streptomyces* isolates[34]. Moreover, the application of this toolkit was extended to other actinomycetes, such as *Micromonospora chersina*[35], *Corynebacterium glutamicum*[36], *Saccharopolyspora erythraea*[37], and *Verrucosispora sp.* MS100137[38]. We furthermore believe that this toolkit can be applied to additional species with minimal effort, for example, by exchanging the replicon and/or related promoters.

## Applications with CRISPR–Cas9-based plasmids

In previous work, we demonstrated that by using the plasmid pCRISPR–Cas9 from the CRISPR–Cas9 toolkit in the WT *S. coelicolor* A3(2) strain and many other streptomycetes[9], a library of differently sized deletions around the target site can be generated that lacks the LigD component because of the 'incomplete' non-homologous end joining (NHEJ) pathway.

When adding an ~2-kb editing template composed of 1 kb each up- and downstream of the gene of interest to the pCRISPR–Cas9 plasmid, we can obtain an in-frame deletion event with nearly 100% editing efficiency[9]. By including other DNA sequences, such as gene(s) of interest or promoters in the editing template, the system can also provide highly specific chromosomal insertions. This concept was reported by Zhang et al[39].

As all CRISPR plasmids described in this protocol follow the same design principle, the Csy4-mediated sgRNA multiplexing strategy[15] initially established for CRISPR-BEST can also be integrated into the original CRISPR–Cas9 toolkit. However, one needs to keep in mind that if multiple DSBs are simultaneously introduced into the genome of streptomycetes, the genomes may become unstable and thus the genome structure analysis and a genome-wide off-target evaluation (Step 34C) should be carried out for positive clones.

## Applications with CRISPR-BEST based plasmids

Here, we provide a protocol for one application of CRISPR-BEST, the inactivation of coding genes by introducing a STOP codon into streptomycetes. The application of the CRISPR-BEST toolkit can of course be easily extended to do in vivo protein engineering by customized amino acid substitutions.

## Strengths and limitations of the presented methods

The protocols facilitate highly efficient, precise, and rapid generation of desired genetic modifications in streptomycetes, showing the following advantages over the conventional double crossover–based methods, including PCR-targeting-based strategies[22]: (i) the protocol is easy to perform; in most cases only one highly efficient cloning step is required to construct a functional CRISPR plasmid; (ii) the protocol does not require genome-mapped cosmid clones of the strain to be engineered (which is typically required in the PCR-targeting approach); (iii) the protocol is relatively fast (~10 d for inactivation of a gene); (iv) the protocol is versatile and covers various genetic manipulations, including in-frame knockout, in-frame knockin, introduction of indels, single-amino-acid exchange and target gene knockdown; (v) the protocol is capable of multiplexed genome editing with a single plasmid targeting multiple genes of interest simultaneously. This has been so far been demonstrated only in base editing applications using pCRISPR–cBEST[15], but it can be easily applied to CRISPRi applications.

As with any other protocol using Cas9-based CRISPR genome editing technologies, there are certain limitations: the main concern is off-target effects. There are two typical off-target effects: One causes visible phenotype changes, which can therefore be easily ruled out. The other does not link to any phenotype changes, which requires additional effort (e.g., whole-genome sequencing) to identify. Because the Cas9 protein is an endonuclease, the expression of active nuclease proteins such as Cas9 or Cas12a (Cpf1) has been reported to be toxic[40,41] to some organisms, because of unspecific targeting causing undesired DSBs. If these are repaired by the cell, they can cause off-target effects. This problem can be addressed in different ways, for example, by using CRISPR-based approaches that do not require full endonuclease activity (that is, CRISPR-base editing[26–28] and CRISPR-prime editing[42]), but we highly recommend evaluating off-target effects by whole-genome sequencing.

In addition to these issues affecting CRISPR applications in general, several specific features of streptomycetes must be considered: for example, the >70% GC content makes routine molecular biological operations more difficult than for organisms with lower GC content. The linear chromosome of streptomycetes is relatively unstable and can tolerate large deletions and genome rearrangements without displaying severe phenotypic effects under laboratory conditions. Therefore, any genetic engineering method involving DNA DSBs (e.g., introduced by *I-SceI* or CRISPR–Cas9), can cause severe off-target effects, such as genome deletions and rearrangements[15,24,25], which need to be thoroughly investigated by whole-genome sequencing after the genome editing procedure.

Most streptomycetes are compatible with the CRISPR plasmids presented here, meaning the plasmids can replicate, the resistance genes successfully confer antibiotic resistance in the target strain, and the promoters used to control Cas9/Cas9 variants and sgRNA transcription are compatible with the machinery of the target strain. However, there may be individual strains or closely related genera that may not accept the pSG5 replicon and/or the *tipA* promoter used. In these specific cases, a simple solution is to exchange the plasmid backbone and/or promoter.

## Experimental design

### Plasmid design

Given the lack of efficient replicons and selection markers for streptomycetes[21], we inserted all essential components into a single pSG5 replicon-based plasmid backbone[43], making an 'all-in-one'

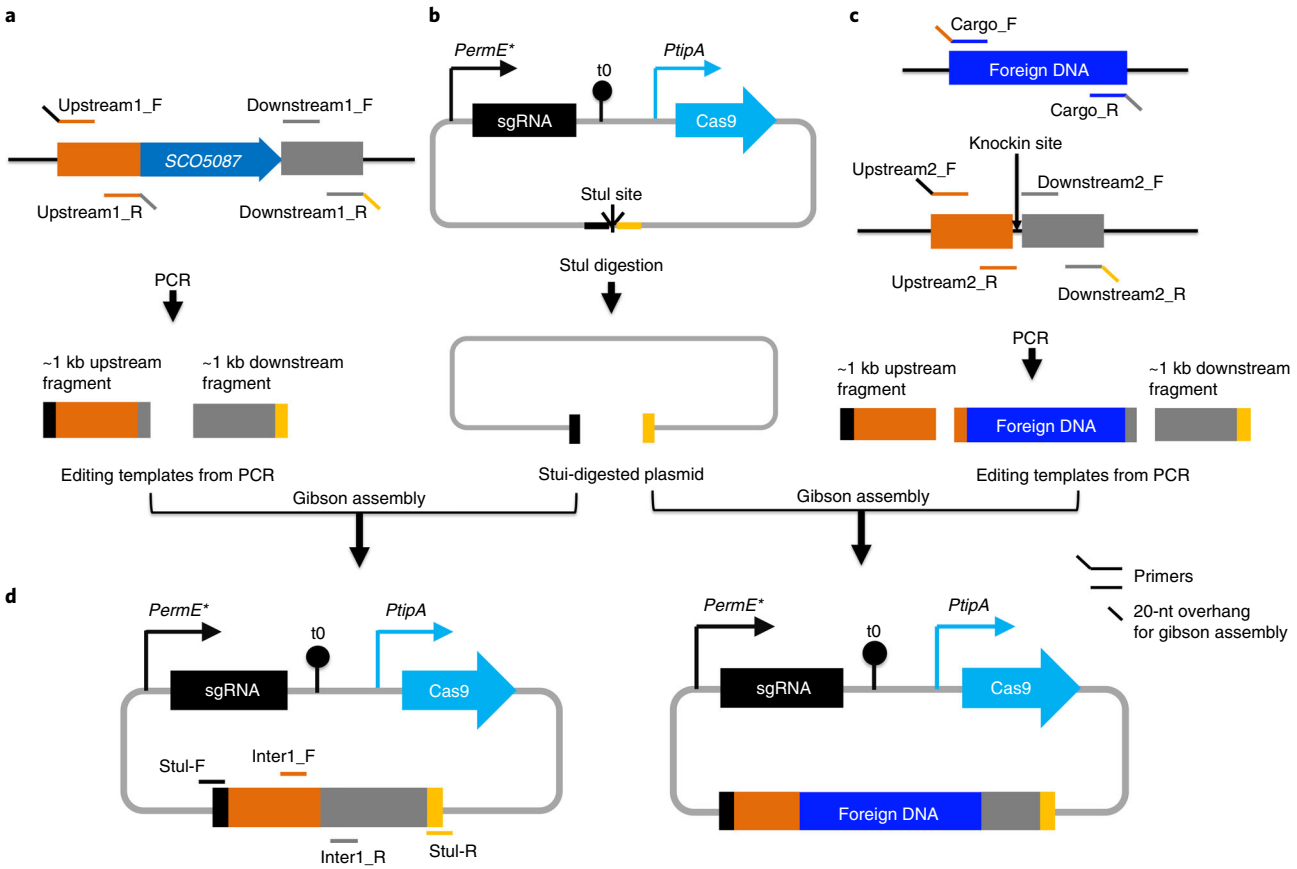


**Fig. 5 | An overview of CRISPR–Cas9 plasmid construction for in-frame deletion or insertion of foreign DNA. a**, Design primers and then PCR amplify ~1 kb up- and downstream DNA fragments of the target gene (*SCO5087* gene is used as an example[9]). For Gibson assembly, the PCR product of the upstream fragment contains 20-bp overhangs from the CRISPR–Cas9 plasmid (shown in black) and the downstream fragment; the PCR product of the downstream fragment contains a 20-bp overhang from the CRISPR–Cas9 plasmid[9] (shown in yellow). **b**, Linearize the desired CRISPR–Cas9 plasmid correctly assembled to contain the sgRNA cassette (from Step 19) by StuI digestion. **c**, Design primers and then PCR-amplify the DNA of interest together with ~1 kb up- and downstream DNA fragments of the knockin site. For Gibson assembly, the PCR products of the up- and downstream fragments each contain a 20-bp overhang from the CRISPR–Cas9 plasmid (shown in black and yellow, respectively), whereas the PCR product of the DNA of interest contains 20-bp overhangs from the up- and downstream fragments. **d**, Use Gibson assembly to assemble fragments from **a** and **b** to construct the desired CRISPR–Cas9 plasmid for in-frame deletion[9], or from **b** and **c** for DNA-of-interest knockin. Primer sets of inter1_F and inter1_R and StuI-F and StuI-R are used for assembly validation. *PtipA*, thiostrepton-inducible promoter; *PermE**, a widely used constitutive promoter; t0, terminator; StuI site: the restriction enzyme StuI recognition and cleavage site.

system. Depending on the specific protocol (Fig. 4), these components include the Cas9/dCas9/Cas9n protein, deaminases, sgRNA, DNA repair components, editing templates, and sgRNA processing machinery. Only one cloning step, to achieve sgRNA assembly, is required to use most of the CRISPR plasmids presented in this protocol (however, an additional cloning step is required for editing template assembly for in-frame deletion/insertion applications). Initially, a NcoI-SnaBI (Eco105I)-based digestion–ligation protocol and a USER-cloning protocol were established for sgRNA assembly[9,16]. To further simplify the protocol and increase the cloning efficiency, an easily scalable PCR- and digestion-free ssDNA oligonucleotide bridging system was set up and used for all described protocols here (Fig. 1). Because the pSG5[43] replicon used is thermo-instable, the plasmids can be cured (eliminated) relatively easily by increasing the incubation temperature and reused repetitively.

### Editing template design

Editing templates are required only for in-frame deletion or for insertions of foreign DNA (Fig. 5). For in-frame deletion, two DNA fragments (one ~1-kb fragment upstream of the targeted DNA and one ~1-kb fragment downstream of the targeted DNA) must be inserted into the pCRISPR–Cas9 plasmid (Fig. 5a,b); whereas for inserting foreign DNA, three DNA fragments (one ~1-kb fragment

upstream of the knockin site, the DNA to be inserted, and one ~1-kb fragment downstream of the knockin site) must be inserted into the pCRISPR–Cas9 plasmid (Fig. 5b,c).

### Multiplexed editing

To further extend the ability of multiplexed editing with the CRISPR toolkits, a Csy4-based sgRNA self-processing machinery[17] was cloned into the 'all-in-one' CRISPR plasmids[15]. Although the prototype multiplexing system was successfully demonstrated only in *S. coelicolor* and *S. griseofuscus* with pCRISPR–cBEST[15], it is reasonable to assume that it would be possible to extend this system to applications using the CRISPRi tool; however, it would be more difficult to apply to the active Cas9-based tools because of the challenges associated with introduction of one, yet alone multiple, DSBs. A Golden Gate assembly method was established for assembling multiple sgRNAs; the rules of primer design are as follows: The overhang of the forward primers needs to consist of (from 5′ to 3′) a random 5-nt adaptor (e.g., GATCG or GATCA); the BsaI recognition site (GGTCTCN); CATG, which after digestion matches the NcoI restriction site of the plasmid backbone (only for the first sgRNA); a 28-nt Csy4 recognition site (only for the first sgRNA); a 20-nt protospacer (from Step 7). The overhang of the reverse primers needs to consist of (from 5′ to 3′) a random 5-nt adaptor (e.g., GATCG), the BsaI recognition site (GGTCTCN), the first 4 nt of the next downstream sgRNA, or CTAG for the last sgRNA in the array, which after digestion matches the NheI overhang of the plasmid backbone (Box 1).

### sgRNA design

To provide a flexible sgRNA design platform, we developed the protospacer identification program CRISPy-web[18,19] (https://crispy.secondarymetabolites.org), which offers a user-friendly web interface and the ability to upload user-specific genome sequences in which to find targets. To support the applications of the CRISPR-BEST toolkit, we recently updated the software[19], which now provides additional options to specifically design sgRNAs for base editing applications. Because different sgRNAs typically have different targeting efficiencies, we recommend targeting at least three different loci per gene with different sgRNAs.

### Modifications to increase efficiency

To reduce the experimental burden, we further established an easy method for *Streptomyces* colony PCR to avoid massive genomic DNA preparation, improved the classic spore conjugation method to increase the transformation efficiency, and modified the traditional protocol for making highly electro-competent *E. coli* ET12567/pUZ8002 (ET) cells[44], which to our knowledge are not commercially available to date.

### Controls

We recommend including two sets of controls: one is the empty plasmid control (no 20-bp protospacer cloned upstream of the sgRNA scaffold), which is involved in interspecies conjugation (Steps 21–33); and the other one is the WT strain control, which is involved in the editing evaluation (Step 34, Sanger sequencing and Illumina sequencing). The empty plasmid control indicates conjugation efficiency and the editing efficiency of the selected spacers. The WT strain control can be used to determine both on-target and off-target editing effects by confirming the exact sequence of the WT strain at the time of the experiment.

## On-target evaluation of CRISPRi applications

Unlike the other CRISPR applications described in this protocol, the use of CRISPR interference[45] (CRISPRi)/CRISPR–dCas9 will not cause chromosomal changes; it will block only the transcription process, via prevention of transcription initiation or transcription elongation steps by the nuclease activity–defective Cas9 (dCas9)–sgRNA–target-DNA complex (Fig. 2). Therefore, Sanger sequencing or whole-genome sequencing cannot evaluate its effects. It requires validation from the transcriptional level and/or endpoint product level of a pathway. Because the protocol presented here aims to affect the endpoint production quantity of the final molecule that is synthesized by a metabolic pathway, we describe the protocol for actinorhodin detection in *S. coelicolor*[9] as a method of validating the CRISPRi effect.

**Box 1 | An example of a three-sgRNA multiplexing array design**

The three-spacer sgRNA array[15] was designed to simultaneously target the three key enzymes from three biosynthetic gene clusters in *S. coelicolor* A3(2): *SCO5087* from the actinorhodin gene cluster (BLUE), *SCO3230* from the calcium-dependent antibiotic gene cluster (CDA), and *SCO5892* from the undecylprodigiosin gene cluster (RED). The sgRNA array is organized by the order of BLUE-CDA-RED.
In the sequences below, the restriction enzyme (RE) handle is in black, the BsaI recognition site is in lowercase letters, the 28-nt Csy4 recognition site is in blue, the 82-nt sgRNA handle is in red, the 20-nt spacer is in green, and the 4-nt overhang is underlined.

- sgRNA-BLUE:

  GATCGggtctccCATGGTTCACTGCCGTATAGGCAGCTAAGAAACCGTTCACAGGTCGCG
  GCGGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAA
  AAAGTGGCACCGAGTCGGTGCTTTTTTGTTCACTGCCGTATAGGCAGCTAAGAAAGCG
  GtgagaccCGATC

- sgRNA-CDA:

  GATCGggtctcaGCGGCGAACCAGCCCATCATGTTTTAGAGCTAGAAATAGCAAGTTAAAA
  TAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTGTTCAC
  TGCCGTATAGGCAGCTAAGAAACCCCtgagaccCGATC

- sgRNA-RED:

  GATCAggtctcaCCCCCAGGACGTGGAACAGAGTTTTAGAGCTAGAAATAGCAAGTTAAAA
  TAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTGTTCAC
  TGCCGTATAGGCAGCTAAGAAACTAGcgagaccTGATC

- sgRNA-BLUE-F: 5′-GATCAGGTCTCGCATGGTTCACTGCCGTATAGGCAGCTAAGAAA
  GTTCACAGGTCGCGGCGGGTTTTAGAGCTAGAAATAGCAAGT-3′

- sgRNA-BLUE-R: 5′-GATCGGGTCTCACCGCTTTCTTAGCTGCCTATACGG-3′

- sgRNA-CDA-F: 5′-GATCGGGTCTCAGCGGCGAACCAGCCCATCATGTTTTAGAGCTAG
  AAATAGCAAGT-3′

- sgRNA-CDA-R: 5′-GATCGGGTCTCAGGGGTTTCTTAGCTGCCTATACGG-3′

- sgRNA-RED-F: 5′-GATCAGGTCTCACCCCCAGGACGTGGAACAGAGTTTTAGAGCTAG
  AAATAGCAAGT-3′

- sgRNA-RED-R: 5′-GATCAGGTCTCGCTAGTTTCTTAGCTGCCTATACGG-3′

## Materials

### Biological materials

**! CAUTION** Diligently follow all regulations/safety instructions of your institute/university/local government when carrying out experiments with microorganisms.

- One Shot Mach1 T1 phage-resistant chemically competent *E. coli*, F$^-$ φ80(*lacZ*)ΔM15 Δ*lacX74 hsdR* ($r_K^-m_K^+$) Δ*recA1398 endA*1 *tonA* (Thermo Fisher Scientific, cat. no. C862003) **! CAUTION** Competent cells should be stored at −80 °C before use. The cells are stable for up to 6 months at −80 °C.
- *E. coli* DH5α, F$^-$ *endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG purB20* φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, *hsdR17*($r_K^-m_K^+$), λ$^-$. (Thermo Fisher Scientific, cat. no. 18265017). These cells are used for maintaining the pJET1.2–sgRNA handle.

- *E. coli* ET12567/pUZ8002 (ET; ref. [44]), F⁻ *dam*-13::Tn9 *dcm*-6 *hsdM hsdR zjj-202*::Tn10 *recF143 galK2 galT22 ara-14 lacY1 xyl-5 leuB6 thi-1 tonA31 rpsL136 hisG4 tsx-78 mtl-1 glnV44*, $Cm^r/Km^r$ (Life Science Market, cat. no. S0052) **! CAUTION** Maintaining *E. coli* ET12567 cells requires both chloramphenicol and kanamycin, because *E. coli* ET12567 has a chloramphenicol-resistance marker and the carrier pUZ8002 has a kanamycin-resistance marker.
- *Streptomyces coelicolor* A3(2) (ref. [46]) (DSMZ, cat. no. 40783) **! CAUTION** Over the years, the *S. coelicolor* WT maintained in our lab has accumulated several SNPs and a deletion of an integrated plasmid[9,15]. Therefore, we highly recommend that users sequence their own WT/parental strain when evaluating the genome editing results.

## Reagents

- $ddH_2O$ (Milli-Q filtered water)
- FastDigest BsaI/Eco31I (Thermo Fisher Scientific, cat. no. FD0294)
- FastDigest Eco147I (StuI; Thermo Fisher Scientific, cat. no. FD0424)
- BsaI-HF v2 kit (New England BioLabs, cat. no. R3733S)
- FastDigest NcoI (Thermo Fisher Scientific, cat. no. FD0573)
- FastDigest NheI (Thermo Fisher Scientific, cat. no. FD0973)
- FastAP thermosensitive alkaline phosphatase (Thermo Fisher Scientific, cat. no. FD0652)
- T4 ligase (5U; Thermo Fisher Scientific, cat. no. EL0014)
- Lysozyme from chicken egg white (Sigma-Aldrich, cat. no. L6876-10G)
- Protease K (>600 U/mL, ~20 mg/mL; Thermo Fisher Scientific, cat. no. EO0491)
- Phusion High-Fidelity PCR Master Mix with HF Buffer (Thermo Fisher Scientific, cat. no. F531L)
- Phusion High-Fidelity PCR Master Mix with GC Buffer (Thermo Fisher Scientific, cat. no. F532L)
- OneTaq 2× Master Mix with standard buffer (New England BioLabs, cat. no. M0482L)
- NEBuilder HiFi DNA Assembly Master Mix (New England BioLabs, cat. no. E2621L)
- Q5 High-Fidelity 2× Master Mix (New England BioLabs, cat. no. M0492L)
- KAPA HyperPlus Kit (Roche, cat. no. 07962428001)
- NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel, cat. no. 740609.250)
- NucleoSpin Plasmid EasyPure Kit (Macherey-Nagel, cat. no. 740727.250)
- Blood & Cell Culture DNA Midi Kit (Qiagen, cat. no. 13343)
- Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific, cat. no. Q32853)
- Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, cat. no. Q32854)
- Mix2Seq Kit Overnight (Eurofins Genomics)
- Dimethyl sulfoxide (DMSO; Sigma-Aldrich, cat. no. 472301-500ML)
- Ethanol, absolute (VWR, cat. no. 20821.310) **! CAUTION** Absolute ethanol is highly flammable. Store it in a fireproof cabinet at room temperature; use it with caution and keep away from open flame. Please use it before the expiration date noted by the provider.
- Ethanol (≥70% (vol/vol); VWR, cat. no. 83801.290)
- Glycerol (≥99.5% (wt/vol); Sigma-Aldrich, cat. no. G9012-1GA)
- Isopropanol (Sigma-Aldrich, cat. no. I9516) **! CAUTION** Absolute isopropanol is highly flammable. Store it in a fireproof cabinet at room temperature; use it with caution and keep away from open flame. Please use it before the expiration date noted by the provider.
- Ampicillin sodium salt (Sigma-Aldrich, cat. no. A0166-25G)
- Apramycin sulfate salt (Sigma-Aldrich, cat. no. A2024-5G)
- Chloramphenicol (Sigma-Aldrich, cat. no. C1919-25G)
- Kanamycin sulfate (Sigma-Aldrich, cat. no. K1377-25G)
- Nalidixic acid (Sigma-Aldrich, cat. no. N8878-5G)
- Thiostrepton (Sigma-Aldrich, cat. no. T8902-1G)
- LB medium (Lennox; Sigma-Aldrich, cat. no. L3022-1KG)
- LB medium with agar (Miller; Sigma-Aldrich, cat. no. L3147-1KG)
- ISP2 agar (ISP medium 2; BD Difco, cat. no. DF0770-17-9)
- DNA gel loading dye (6x; Thermo Fisher Scientific, cat. no. R0611)
- DNA stain (RedSafe; iNtRON Biotechnology, cat. no. 21141) **! CAUTION** Although this product is claimed to be without mutagenesis concerns, we highly recommend using proper protection when handling it.
- GeneRuler 1-kb DNA ladder (Thermo Fisher Scientific, cat. no. SM0311)
- GeneRuler 100-bp DNA ladder (Thermo Fisher Scientific, cat. no. SM0242)
- GeneRuler 50-bp DNA ladder (Thermo Fisher Scientific, cat. no. SM0372)

- Trizma base (Sigma-Aldrich, cat. no. 93362-1KG)
- NEBuffer 2 (10x; New England BioLabs, cat. no. B7002S)
- Sodium hydroxide (NaOH; pellets; Honeywell, cat. no. 30620-1KG)
- Sodium chloride (NaCl; Honeywell, cat. no. 13423-1KG-R)
- Acetic acid (Honeywell, cat. no. 33209-4x2.5L-M) **! CAUTION** Acetic acid is flammable, corrosive, and volatile. Use it inside a chemical fume hood with caution and keep away from open flame.
- Hydrochloric acid (HCl; VWR, cat. no. 20252.290) **! CAUTION** HCl is highly corrosive and volatile. Use it with caution inside a chemical fume hood.
- Magnesium chloride ($MgCl_2$; Sigma-Aldrich, cat. no. M4880-100G)
- Oxoid yeast extract powder (Thermo Fisher Scientific, cat. no. LP0021B)
- Malt extract (Sigma-Aldrich, cat. no. 70167-500G)
- Dextrose (D-Glucose; Sigma-Aldrich, cat. no. D9434-1KG)
- Agar (Sigma-Aldrich, cat. no. 05040-1KG)
- D-Mannitol (≥98% (wt/wt); Sigma-Aldrich, cat. no. M4125-1KG)
- Hensel organic soy flour (fat reduced; Violey, product no. 02004307)
- Standard agarose, type LE (BioNordika, cat. no. BN50004)
- Trizma hydrochloride (Tris-HCl; Sigma-Aldrich, cat. no. T3253-500G)
- EDTA (ethylenediaminetetraacetic acid disodium salt dihydrate; Sigma-Aldrich, cat. no. E5134-500G)
- Tween 20 (for molecular biology, viscous liquid; Sigma-Aldrich, cat. no. P9416-100ML)
- Triton X-100 (Thermo Fisher Scientific, cat. no. AC215682500)
- Guanidine hydrochloride (guanidine HCl; Sigma-Aldrich, cat. no. G3272-500G)
- CloneJET PCR Cloning Kit (Thermo Fisher Scientific, cat. no. K1231)
- Tryptone (Sigma-Aldrich, cat. no. T7293-1KG)
- Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$, Sigma-Aldrich, cat. no. 63138-250G)
- Potassium chloride (KCl, Sigma-Aldrich, cat. no. P9541-1KG)
- Liquid nitrogen (local supplier)
- Sucrose (Sigma-Aldrich, cat. no. S0389-1KG)

**Primers**

- StuI-F: 5′- GACAATGACAACAACCATCGCC-3′ (Integrated DNA Technologies (IDT), custom order)
- StuI-R: 5′- GGGAAGTCGTCGCTCTCTGG-3′ (IDT, custom order)
- sgRNA-TEST-F: 5′-AATTGTACGCGGTCGATCTT-3′ (IDT, custom order)
- sgRNA-TEST-R: 5′-TACGTAAAAAAAGCACCGAC-3′ (IDT, custom order)
- Cas9-C-terminal-TEST: 5′-GACCCTGATCCACCAGAGCA-3′ (IDT, custom order)
- Upstream1_F: 5′-**TCGTCGAAGGCACTAGAAGG**CATCCGCTGAACGAGACCC-3′ (sequence in bold represents 20-nt overhang with CRISPR–Cas9 plasmid; IDT, custom order)
- Upstream1_R: 5′-**GCTCACGTCGAAGCGGGTGA**CCACGCAGGACTCCGAAGTC-3′ (sequence in bold represents 20-nt overhang with the downstream1 fragment; IDT, custom order)
- Downstream1_F: 5′-TCACCCGCTTCGACGTGAG-3′ (IDT, custom order)
- Downstream1_R: 5′-**GGTCGATCCCCGCATATAGG**TTCGCCGAGCACCAGGTC-3′ (sequence in bold represents 20-nt overhang with CRISPR–Cas9 plasmid; IDT, custom order)
- Inter1_F: 5′-TTGTTCACCAGCACGTCGAC-3′ (IDT, custom order)
- Inter1_R: 5′-AAGTCGAACCCACCGCAGG-3′ (IDT, custom order)

**Plasmids**

- pCRISPR–Cas9 (RRID: Addgene_125686)
- pCRISPR–dCas9 (RRID: Addgene_125687)
- pCRISPR–Cas9–ScaligD (RRID: Addgene_125688)
- pCRISPR–cBEST (RRID: Addgene_125689)
- pCRISPR–aBEST (RRID: Addgene_131464)

## Equipment

- mLINE pipettes (0.1–3, 0.5–10, 5–100, and 100–1,000 µL; Sartorius, cat. nos. 725010, 725120, 725130, and 725070)
- Optifit refill tips (10, 200, and 1,000 µL; Sartorius, cat. nos. 790012, 790202 and 791002)
- Minisart syringe filter (0.2 µm; single use; nonpyrogenic; Sartorius, cat. no. 50192103)
- Quintix lab scale (Sartorius, cat. no. QUINTIX213-1S)
- Pipetboy acu 2 (Integra Biosciences, item no. 155017)

- Serological pipettes (sterile, CellStar, 1, 10, and 25 mL; Greiner Bio-One, cat. nos. 604181, 608180, and 760180)
- Bacteriological Petri dishes (94 mm × 16 mm; not tissue culture treated, without vents, aseptic; LabSolute, cat. no.7696401)
- Deep-well plates (96-well, VWR, cat. no. 82006-448)
- Mini Centrifuge (VWR, cat. no. 76269-064)
- Shake flasks (250 mL; wide-mouth flasks; VWR, cat. no. 214-1132)
- C1000 Touch thermal cycler (Bio-Rad, cat. no. 1851148)
- S1000 thermal cycler (Bio-Rad, cat. no. 1852196)
- MicroPulser electroporator (Bio-Rad, cat. no. 1652100)
- Molecular imager (Gel Doc XR+ Gel Documentation System with Image Lab software; Bio-Rad, cat. no. 1708195)
- Gel electrophoresis tank and power source (Bio-Rad Mini-Sub and Wide Mini-Sub Cell GT Cells; Bio-Rad, cat nos. 1704466 and 1704468EDU; Bio-Rad PowerPac basic power supply; Bio-Rad, cat. no. 1645050)
- Invitrogen Safe Imager 2.0 (Invitrogen cat. no. G6600EU)
- Floor centrifuge (Heraeus Multifuge X3 FR; Thermo Fisher Scientific, cat. no. 75004500)
- Rotors (Fiberlite F14-6x250 LE and Fiberlite F13-14x50cy LE; Thermo Fisher Scientific, cat. nos. 75003661 and 75003662)
- Benchtop centrifuge (Heraeus Fresco17; Thermo Fisher Scientific, cat. no. 75002420)
- Desktop centrifuge (Micro Star 17; VWR, cat. no. 521-1646P)
- Desktop microcentrifuge (Mini Star Silverline; VWR, cat. no. 521-2845P)
- Incubator (Labnet 311DS; Labnet International, cat. no. I5311-DS)
- Shaker (New Brunswick Innova 42/42R; Eppendorf, cat. no. M1335-0002)
- Polypropylene centrifuge bottles (250 mL; Corning, cat. no. 431841)
- Heating block (Eppendorf Thermomixer Comfort; Eppendorf, cat. no. 5355 000.011)
- UV spectrophotometer (NanoDrop; Thermo Fisher Scientific, model no. 2000)
- Qubit 2.0 Fluorometer (Thermo Fisher Scientific, cat. no. Q32866)
- Qubit assay tubes (Thermo Fisher Scientific, cat. no. Q32856)
- Gene Pulser Cuvette/*E. coli* cuvette (0.1-cm electrode; Bio-Rad, cat. no. 165-2089)
- Magnetic stirrer (RCT basic; IKA, cat. no. 0003810000)
- Magnetic stir bar (IKAFLON 155; IKA, item. no. 0001129000)
- Benchtop autoclave (Certoclav Multicontrol; CertoClav Sterilizer, cat. no. 8510298)
- Safe-Lock microcentrifuge tubes (1.5 mL; Eppendorf, cat. no. 0030120086)
- 50 mL Falcon tubes (polypropylene; 50 mL; Sarstedt, cat. no. 62547254)
- Falcon tubes (CellStar, 15 mL, polypropylene; Greiner Bio-One, cat. no. 188271)
- 8-strip PCR tubes (Sarstedt, cat. no. 72.985. 002)
- Flat-cap well-sealing strips (Cell Projects, cat. no. FC-08-CC/CP)
- Inoculating loop (10 μL; Sigma-Aldrich, cat. no. I8388-500EA)
- Inoculation spreader (Sarstedt, cat. no. 86.1569.005)
- pH meter (827 pH lab; Metrohm, cat. no. 2.827.0214)
- Laminar flow hoods (biological safety cabinets, class 2; Mars-900 or Mars-1200; LaboGene, cat. nos. 9.001.023.000 or 9.001.020.000)
- Heratherm incubator (Thermo Fisher Scientific, cat. no. WZ-38800-16)
- Vortex-Genie 2 (Scientific Industries, cat. no. SI-0236)
- CryoPure tubes (1.8 mL, white, nonpyrogenic, noncytotoxic, nonmutagenic; Sarstedt, cat. no. 72.379)
- Optical density (OD) meter (Ultrospec 10 Classic; Biochrom, cat. no. 84-741)
- OD cuvettes (Semi-micro cuvette, polystyrene, minimum filling volume 1.5 mL; Brand, cat. no. 759015)
- Cotton swabs (Matas, cat. no. 732634)
- Cotton pads (Matas, cat. no. 731144)
- Wooden toothpicks (Plastico, cat. no. 304)
- Sera-Mag Select (GE Healthcare, cat. no. 29343045)
- Fragment analyzer system (Agilent Technologies, model no. 5300, cat. no. M5311AA)
- Sequencer (Illumina, NextSeq 500 model)
- Ice machine (Scotsman, cat. no. MISTERNAC176)
- Milli-Q water system (Purelab Flex 2, Elga, cat. no. PF2XXXXM1-KIT)
- BlueCap bottles with blue GL45 lids (100, 250, 500, and 1,000 mL; Buch-Holm, cat. nos. 9072331, 9072332, 9072334, and 9072335)

- Parafilm (Sigma-Aldrich, cat. no. P7793-1EA)
- Aluminum foil
- Paper towels
- Autoclave tape
- Tweezers
- Scissors

## Software

- Melting temperature ($T_m$) calculator (NEB, RRID: SCR_017969, https://tmcalculator.neb.com/#!/main)
- CRISPy-web[18,19] (RRID: SCR_017970, https://crispy.secondarymetabolites.org)
- CLC Main Workbench (Qiagen Bioinformatics, RRID: SCR_000354, https://www.qiagenbioinformatics.com/products/clc-main-workbench/)
- Image Lab Software (Bio-Rad, v.6.0.1, RRID: SCR_014210, https://www.bio-rad.com/en-us/product/image-lab-software?ID=KRE6P5E8Z)
- merge-gbk-records (RRID: SCR_017968, https://github.com/kblin/merge-gbk-records)
- AdapterRemoval v.2 (ref. [47]; RRID: SCR_011834, https://github.com/MikkelSchubert/adapterremoval)
- breseq[48] (v.0.33.2, RRID: SCR_010810, https://code.google.com/archive/p/breseq/)
- FastQC (RRID: SCR_014583, http://www.bioinformatics.babraham.ac.uk/projects/fastqc/)
- SnapGene (RRID: SCR_015052, https://www.snapgene.com)

## Reagent setup

### MS agar plates

Make MS (Mannitol Soya Flour) agar plates by mixing well (with a magnetic stirrer) 20 g/L D-mannitol, 20 g/L fat-reduced soy flour, and 20 g/L agar in normal tap water. Adjust the pH to 8 with 1 M NaOH before autoclaving. After autoclaving at 121 °C for 20 min, add pre-autoclaved $MgCl_2$ to a final concentration of 10 mM; then use a magnetic stirrer to mix until the temperature naturally lowers to ~50 °C for pouring into Petri dishes. The MS plates can be stored at 4 °C for 1 month **! CAUTION** Tap water from different suppliers might affect the efficiency of conjugation and/or sporulation of some streptomycetes; different fat compositions of soy flour might also affect the efficiency of conjugation and/or sporulation of some streptomycetes.

### ISP2 plates for exconjugant screening

Because all plasmids described in this protocol share the same selection marker of apramycin, we used the same type of antibiotic-containing (selective) ISP2 plates for screening and maintaining the exconjugants. We refer to ISP2 plates without supplemented antibiotics as 'nonselective ISP2' plates; the same rule applies to ISP2 broth. Dissolve premixed ISP2 agar powder in normal tap water and adjust the pH to 7.4 with 1 M NaOH before autoclaving. After autoclaving (121 °C, 20 min), naturally cool down the medium to ~50 °C at room temperature, add 50 μg/mL apramycin and 50 μg/mL nalidixic acid (optionally, add 0.5 μg/mL thiostrepton for induction), mix well and then pour into Petri dishes. Plates can be stored at 4 °C for up to 1 month.

### LB plates

Make LB plates by dissolving premixed LB agar powder in normal tap water and adjust the pH to 7.0 with 1 M NaOH before autoclaving. After autoclaving (121 °C, 20 min), naturally cool down the medium to ~50 °C at room temperature, add antibiotics when needed, mix well and pour into Petri dishes. LB plates with antibiotics are called selective LB plates (otherwise, nonselective LB plates); the same rule applies to LB broth. Plates can be stored at 4 °C for up to 1 month.

### LB broth

Make LB broth by dissolving premixed LB broth powder in normal tap water and adjust the pH to 7.0 with 1 M NaOH before autoclaving. After autoclaving (121 °C, 20 min), naturally cool down the medium to ~50 °C at room temperature and add antibiotics when needed. LB broth can be stored at 4 °C for up to 3 months. **! CAUTION** Thiostrepton is not always required for induction, because the *tipA* promoter is leaky in many strains, and the leaky expression of its controlled components is usually enough to achieve the desired genome editing events **▲ CRITICAL** Because streptomycetes have a relatively low growth rate, nalidixic acid is used for counterselecting the *E. coli* used for interspecific conjugation.

**ISP2 broth for routine maintenance of streptomycetes**
Make ISP2 broth by mixing 4 g/L of yeast extract, 10 g/L of malt extract, and 4 g/L of dextrose in normal tap water with a magnetic stirrer. Adjust the pH to 7.4 with 1 M NaOH before autoclaving (121 °C, 20 min). Autoclaved ISP2 broth can be stored at 4 °C for up to 3 months.

**YEME (yeast extract–malt extract) broth with 3.4 % (wt/vol) sucrose**
Make YEME broth by mixing 3 g/L of yeast extract, 3 g/L of malt extract, 5 g/L of peptone, 10 g/L of glucose and 34 g/L of sucrose in normal tap water with a magnetic stirrer. Adjust the pH to 7.2 with 1 M NaOH before autoclaving (121 °C, 20 min). Autoclaved YEME broth can be stored at 4 °C for up to 3 months.

**Ampicillin stock solution (100 mg/mL)**
Use an electronic scale to weigh 1,000 mg ampicillin sodium salt and dissolve it in 10 mL $ddH_2O$. Filter the solution with a 0.2-μm filter in a laminar flow hood, and then make aliquots in sterilized 1.5-mL Eppendorf tubes. The stock can be stored at −20 °C for up to 1 year.

**Apramycin stock solution (50 mg/mL)**
Use an electronic scale to weigh 500 mg apramycin sulfate salt and dissolve it in 10 mL $ddH_2O$. Filter the solution with a 0.2-μm filter in a laminar flow hood and then make aliquots in sterilized 1.5-mL Eppendorf tubes. The stock can be stored at −20 °C for up to 1 year.

**Chloramphenicol stock solution (25 mg/mL)**
Use an electronic scale to weigh 250 mg kanamycin sulfate and dissolve it in 10 mL absolute ethanol. Filter the solution with a 0.2-μm filter in a laminar flow hood and then make aliquots in sterilized 1.5-mL Eppendorf tubes. The stock can be stored at −20 °C for up to 1 year.

**Kanamycin stock solution (50 mg/mL)**
Use an electronic scale to weigh 500 mg kanamycin sulfate and dissolve it in 10 mL $ddH_2O$. Filter the solution with a 0.2-μm filter in a laminar flow hood and then make aliquots in sterilized 1.5-mL Eppendorf tubes. The stock can be stored at −20 °C for up to 1 year.

**Nalidixic acid stock solution (50 mg/mL)**
Use an electronic scale to weigh 500 mg nalidixic acid and dissolve it in 10 mL 0.1 M NaOH. Filter the solution with a 0.2-μm filter in a laminar flow hood and then make aliquots in sterilized 1.5-mL Eppendorf tubes. The stock can be stored at −20 °C for up to 1 year **! CAUTION** NaOH is corrosive. Use it with caution.

**Thiostrepton stock solution (5 mg/mL)**
Use an electronic scale to weigh 10 mg thiostrepton and dissolve it in 2 mL DMSO. Filter the solution with a 0.2-μm filter in a laminar flow hood and then make aliquots in sterilized 1.5-mL Eppendorf tubes. The stock can be stored at −20 °C for up to 1 year.

**SOC broth for *E. coli* transformation**
Make SOC broth by mixing 20 g/L of tryptone, 5 g/L of yeast extract, 4.8 g/L of $MgSO_4 \cdot 7H_2O$, 3.603 g/L of dextrose, 0.5 g/L of NaCl, and 0.186 of g/L KCl in normal tap water using a magnetic stirrer. Adjust the pH to 7.0 with 1 M NaOH before autoclaving (121 °C, 20 min). Autoclaved SOC broth can be stored at 4 °C for up to 3 months.

**2× YT broth**
Make 2× YT broth by mixing 16 g/L of tryptone, 10 g/L of yeast extract, and 5 g/L of NaCl in normal tap water using a magnetic stirrer. Adjust the pH to 7.0 with 1 M NaOH before autoclaving (121 °C, 20 min). Autoclaved 2× YT broth can be stored at 4 °C for up to 3 months.

**Glycerol (50% and 10% (vol/vol))**
To make 50% glycerol, mix 100 mL glycerol with 100 mL $ddH_2O$ and autoclave it at 121 °C for 20 min. Make 10% (vol/vol) glycerol by mixing 100 mL of 50% (vol/vol) glycerol with 400 mL of $ddH_2O$. Both glycerol solutions can be stored at room temperature (22–25 °C) for up to 1 year.

**0.5 M EDTA (pH 8.0)**
Use an electronic scale to weigh 93.05 g EDTA, dissolve it in ~400 mL $ddH_2O$, and adjust the pH to 8.0 using NaOH pellets. Top up the solution to a final volume of 500 mL and autoclave it at 121 °C for 20 min. Autoclaved EDTA solution can be stored at 4 °C for up to 3 months.

**50× TAE buffer**
Use an electronic scale to weigh 242 g Trizma base (Tris base); dissolve it in ~700 mL $ddH_2O$ and carefully add 57.1 mL 100% acetic acid and 100 mL 0.5 M EDTA (pH 8.0). Adjust the solution to a final volume of 1 L. The pH does not need to be adjusted and should be about 8.5. Make 1× TAE buffer by mixing 20 mL of 50× TAE buffer (vol/vol) with 980 mL of $ddH_2O$. Both TAE buffers can be stored at room temperature for up to 6 months.

**Buffer B1**
Make Buffer B1 by dissolving 18.61 g EDTA and 6.06 g Trizma base in 800 mL $ddH_2O$. Then add 50 mL 10% (wt/vol) Tween 20 solution and 50 mL 10% (wt/vol) Triton X-100 solution. Adjust the pH to 8.0 with 1 M HCl. Adjust the volume to 1 L with $ddH_2O$. Filter the solution with a 0.2-μm filter in a laminar flow hood. It can be stored at room temperature for up to 1 month.

**Buffer B2**
Make Buffer B2 by dissolving 286.59 g guanidine HCl in 700 mL $ddH_2O$. Add 200 mL of 100% Tween 20. Adjust the volume to 1 L with $ddH_2O$. The pH does not need to be adjusted. Filter the solution with a 0.2-μm filter in a laminar flow hood. It can be stored at room temperature for up to 1 month.

**pJET1.2–sgRNA handle**
Clone the 82-nt sgRNA handle, followed by a 28-nt Csy4 recognition site (GTTTTAGAGCTAGAAAT AGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTT TTTGTTCACTGCCGTATAGGCAGCTAAGAAA) into pJET1.2 (from the CloneJET PCR Cloning Kit)
▲ **CRITICAL** Ampicillin (100 μg/mL) is used to maintain the pJET1.2–sgRNA handle carrying DH5α *E. coli*.

## Procedure

▲ **CRITICAL** All reagents and equipment are used according to the instructions provided by their manufacturers, unless specific modifications are indicated. DNA oligonucleotides are synthesized by IDT, custom DNA fragments that are codon-optimized (optional) are synthesized by GenScript. Diligently follow all waste disposal regulations of your institute/university/local government when disposing of waste materials. Living bacteria-related operations are carried out in a laminar flow hood to avoid contamination. All primer solutions are 20 μM in this protocol, unless otherwise indicated. An overview of the Procedure is shown in Fig. 4.

### sgRNA design ● Timing 30 min

1 Upload the genome of interest. Genome data can be uploaded to CRISPy-web at https://crispy.secondarymetabolites.org/ in GenBank format containing CDS (coding DNA sequence) feature annotations for the genes of interest. This can be achieved manually using option A or directly via combination of CRISPy-web and the antiSMASH genome mining tool[49], using option B.

(A) **Submit genome data to CRISPy-web manually**

(i) Use the 'Browse' button to find the input file on your local machine and hit 'Start' to submit the file to the CRISPy-web server.

**! CAUTION** Gene annotations are required to predict the codon changes introduced during CRISPR-BEST editing, so genome data in FASTA format or without gene annotations are not supported.

**! CAUTION** CRISPy-web is designed to work on single-chromosome, single-contig bacterial genomes. To properly detect off-target matches on multi-contig draft genomes, merge all contigs into a single record (using a tool such as https://github.com/kblin/merge-gbk-records).

**? TROUBLESHOOTING**

(B) **Directly import genome data into CRISPy-web using antiSMASH**

(i) Directly fetch files using the antiSMASH job ID via the 'Get sequence from antiSMASH' tab.

2 Select the region of interest. After a few seconds, CRISPy-web will load the genome data and present the region selection screen, which accepts genomic coordinates, locus tag names, or antiSMASH

region numbers (if antiSMASH has found and annotated any biosynthetic regions of interest) as input. Genomic coordinates are the most flexible form of input. For example, if the region you want to edit spans 20 kb from the base 100,000 bp, you would enter a range of '100000-120000'.

▲ CRITICAL STEP The 'Expert settings' panel allows you to tweak the editing window size and offset from the PAM (protospacer-adjacent motif), but unless you have evidence of the edit window properties being different in your strain of interest, we recommend keeping the defaults.

? TROUBLESHOOTING

3 Click the 'Find targets' button to start the protospacer identification process. Depending on the size of your uploaded genome and the current server load, the scan for protospacers will take several minutes. Once the scan is complete, your selected region is shown on top of a table with detailed protospacer descriptions. Genes are denoted as arrows pointing right for genes on the forward strand and pointing left for genes on the reverse strand. Protospacers are indicated by red boxes above and below the gene arrows, depending on the strand of the protospacer. If your region of interest is relatively large, it might be hard to visually distinguish different protospacers on a single gene. You can zoom into genes of interest by left-clicking on a gene arrow and selecting 'Show results for this gene only'. You can always go back to the overview by using the 'Go back' button on the top left of the screen.

4 Select appropriate CRISPR spacers for the desired application. Initially, CRISPy-web shows all potential protospacers. Because these protospacers are scored by potential off-target calculation, one can directly select the desired spacers here for non–base editing applications. Click the 'CRISPR-BEST mode' button to restrict the view to protospacers that can be used for CRISPR-BEST applications. This filters for protospacers that introduce amino acid changes when used in CRISPR-BEST. Click the 'Show only STOP mutations' button to further restrict the results to only show the protospacers that can be used to introduce stop codons.

? TROUBLESHOOTING

5 To select a protospacer for download, simply click on its table row. The text color will change to blue and the counter on the 'shopping cart' button on the upper right will increase by one. More than one protospacer can be selected this way.

6 Once you finish your selection of protospacers, click the 'shopping cart' button on the top right. You will be shown an overview of selected protospacers.

7 Hit 'Download CSV file' to download the selected protospacers in a format compatible with spreadsheet applications. The 20 nucleotides in the 'Sequence' column will be used for sgRNA assembly in Step 8.

▲ CRITICAL STEP In general, low numbers of off-target hits represent good protospacers. In general, we advise selecting protospacers with no 0-bp mismatches and, if possible, with no 1-bp or 2-bp mismatches for maximum specificity. However, for introducing stop codons, good protospacers strike a balance of a low number of off-target hits and a location as close to the start codon as possible.

▲ CRITICAL STEP Mutations are displayed as WT amino acid, coordinate in the protein sequence, and amino acid after mutation. For example, if the mutation is 'L29F', the leucin at position 29 of the protein will be mutated to a phenylalanine. An amino acid code of '*' indicates a stop codon.

▲ CRITICAL STEP Note that CRISPy-web predicts protospacers that target both DNA strands. CRISPRi, however, works only with sgRNAs binding to the non-template DNA strand (coding strand or sense strand) when targeting within an open reading frame (ORF) or sgRNAs binding to both strands of the promoter region[9,45]. This makes protospacer selection for CRISPRi application a critical step. We recommend selecting protospacers in CRISPy-web that can bind to the non-template DNA strand (also known as the coding strand and sense strand), either binding to a promoter or binding to an ORF region that is close to the start codon.

## Construction and validation of the desired CRISPR plasmid ● Timing 7–16 d

8 Prepare sgRNA cassettes and (optional) editing templates.

Follow option A if you are going to construct single-target CRISPR plasmids and option B if you are going to construct multiple-target CRISPR plasmids. Construction of an editing template (option C), is required only for in-frame deletions or insertions of foreign DNA.

(A) **Preparing 20-nt protospacer-containing ssDNA oligonucleotides for single-target CRISPR plasmid construction**

(i) Design ssDNA oligonucleotides for the single-target CRISPR plasmid construction from the protospacer sequences obtained in Step 7. To facilitate the ssDNA oligonucleotide design for protospacer integration into all single-target CRISPR plasmids, use the following template: CGGTTGGTAGGATCGACGGC-**N20**-GTTTTAGAGCTAGAAATAGC. The bold 'N20' represents the place to insert the custom 20-nt protospacer from Step 7.

(ii) Directly order the ssDNA oligonucleotide from the supplier, for example, IDT, with standard desalting protocol.

(iii) Resuspend the oligonucleotides in 1× NEBuffer 2 at a 100 μM stock concentration.

(iv) Before starting Step 13(A), dilute the oligonucleotides to a working concentration of 0.2 μM, using 1× NEBuffer 2.

**PAUSE POINT** The prepared solutions, together with the stocks, can be stored at −20 °C for up to 6 months.

(B) **Preparing sgRNA cassettes for multiple-target CRISPR plasmid construction**

(i) sgRNA cassette preparation for multiplexing-compatible CRISPR plasmids requires PCR to obtain the cassettes. First, design and order primers for each sgRNA fragment according to the design rules in the 'Experimental design' section (an example is presented in Box 1).

(ii) Carry out a PCR for each sgRNA in a 50-μL reaction, using the pJET1.2–sgRNA handle (Materials) as template, with the following setup and conditions:

| Component | Amount (μL) | Final concentration |
|---|---|---|
| Forward primer (from Step 8B(i)) | 1 | 400 nM |
| Reverse primer (from Step 8B(i)) | 1 | 400 nM |
| Template DNA | 0.5 (~50 ng) | |
| 2× Phusion High-Fidelity PCR Master Mix with HF Buffer | 25 | 1× |
| $ddH_2O$ | 22.5 | |
| Total | 50 (one reaction) | |

| Cycle no. | Denature | Anneal | Extend | Final |
|---|---|---|---|---|
| 1 | 98 °C, 20 s | | | |
| 2–31 | 98 °C, 10 s | 60 °C[a], 30 s | 72 °C, 10 s | |
| 32 | | | 72 °C, 5 min | |
| 33 | | | | 10 °C, hold |

[a]Annealing temperature is from $T_m$ calculator.

**! CAUTION** The extension time is calculated according to the DNA polymerase used, for example, 15–30 s/kb for Phusion High-Fidelity polymerase.

**▲ CRITICAL STEP** We obtained equal efficiencies by using the NEB Q5 High-Fidelity 2× Master Mix Kit and the 2× Phusion High-Fidelity PCR Master Mix with HF Buffer.

**▲ CRITICAL STEP** The annealing temperature ($T_a$) in this work is calculated with a $T_m$ calculator from NEB (https://tmcalculator.neb.com/#!/main). Other similar available $T_m$ calculators can also be used.

(iii) Analyze 5 μL of each PCR reaction (add 1 μL of 6× DNA gel loading dye) along with the GeneRuler 100-bp DNA ladder on an agarose gel (1% (wt/vol)) with 1× TAE buffer. Run the gel at 100 V for 30 min and visualize the bands using a Gel Doc XR+ Gel Documentation System. A band at ~150 bp is expected.

**? TROUBLESHOOTING**

(iv) Purify the positive fragments using a NucleoSpin Gel and PCR Clean-up Kit according to the manufacturer's instructions.

**▲ CRITICAL STEP** The reaction can be directly used for the following digestion–ligation processes; however, we recommend first purifying the fragments using the NucleoSpin Gel and PCR Clean-up Kit according to the manufacturer's instructions.

(v) Measure the concentration of each fragment using a NanoDrop 2000 spectrophotometer.

(vi) Pool all fragments, calculate the volume of each fragment required based on the measured concentrations (from Step 8B(v) and a desired molar ratio of 5:1 (insert/vector).

**▲ CRITICAL STEP** This equation is used for picomole-to-nanogram conversion: Picomoles = (weight in nanograms) × 1,000/(base pairs × 650 Da)

(vii) Digest the sgRNA fragments using FastDigest Eco31I (BsaI). Set up a 20-μL reaction using a mix of 1 μL FastDigest Eco31I and 1 μL FastAP thermosensitive alkaline phosphatase and incubate for 30 min at 37 °C. Heat-inactivate for 10 min at 65 °C.

▲ CRITICAL STEP The amount of FastDigest Eco31I (BsaI) needs to be calculated based on the number of fragments; we recommend using 0.2 µL FastDigest Eco31I for each fragment in a 20-µL reaction. In total, the amount of FastDigest Eco31I should not exceed 1 µL. If one has >5 fragments, we recommend using a NEB BsaI-HF v2 kit to insert the sgRNA array into the pGGA vector included in the kit and then applying a NcoI–NheI double digestion to isolate the pre-assembled sgRNA array.

▲ CRITICAL STEP Because there are many BsaI sites in the pSG5 plasmid backbone, a two-step Golden Gate assembly reaction must be set up to insert the sgRNA fragments into the multiplexing-compatible CRISPR plasmids (Step 13B). Therefore, at this stage, removal of all restriction enzymes by proper heat inactivation or cleanup of the fragments is of great importance.

■ PAUSE POINT The prepared sgRNA fragments can be stored at −20 °C for up to 6 months.

(C) **(Optional) Preparation of editing templates** ● Timing **5–12 d**

▲ CRITICAL This step is required only for creating in-frame deletions and/or insertions of foreign DNA (Fig. 5).

(i) Design and order primer sets that can amplify two ~1-kb DNA fragments flanking the DNA sequence of interest (and, optionally, also a third fragment, the DNA of interest to be inserted in the case of an in-frame insertion; see 'Editing template design' in the 'Experimental design' section for further information) with a 20-nt overhang at the ends of each fragment for subsequent Gibson assembly (Fig. 5). Example primer sequences for constructing the CRISPR–Cas9 plasmid that was used to in-frame-delete gene *SCO5087* (Fig. 5a) in *S. coelicolor*[9] can be found in the 'Reagents' section (Upstream1_F, Upstream1_R, Downstream1_F, and Downstream1_R).

(ii) Use direct *Streptomyces* colony PCR (Step 34B) or a genomic DNA as template in a 50-µL PCR reaction for amplification of each of the required DNA fragments as follows:

| Component | Amount (µL) | Final concentration |
|---|---|---|
| Forward primer (from Step 8C(i)) | 1 | 400 nM |
| Reverse primer (from Step 8C(i)) | 1 | 400 nM |
| Template DNA | 1 (~100 ng if genomic DNA is used) | |
| 2× Phusion High-Fidelity PCR Master Mix with GC Buffer | 25 | 1× |
| DMSO | 1.5 | 3% (vol/vol) |
| $ddH_2O$ | 20.5 | |
| Total | 50 (one reaction) | |

| Cycle no. | Denature | Anneal | Extend | Final |
|---|---|---|---|---|
| 1 | 98 °C, 20 s | | | |
| 2–36 | 98 °C, 10 s | 72 °C[a], 30 s | 72 °C, 30 s | |
| 37 | | | 72 °C, 5 min | |
| 38 | | | | 10 °C, hold |

[a]Annealing temperature is from $T_m$ calculator.

▲ CRITICAL STEP We recommend adding 3% (vol/vol) DMSO if pure genomic DNA is used as the PCR template.

(iii) Analyze 5 µL of each PCR reaction (add 1 µL of 6× DNA gel loading dye) along with the GeneRuler 1-kb DNA ladder on an agarose gel (1%, wt/vol) with 1× TAE buffer. Run the gel at 100 V for 30 min and visualize the bands using a Gel Doc XR+ Gel Documentation System.

? TROUBLESHOOTING

(iv) Purify successfully amplified fragments either by PCR cleanup (if the bands are unique and sharp) or gel purification (if unspecific bands are observed) using a NucleoSpin Gel and PCR Clean-up Kit according to the manufacturer's instructions.

**Box 2 | Inserting editing templates into CRISPR plasmids for in-frame deletion or foreign DNA insertions ● Timing 3–5 d**

**Procedure**

1 Digest the non-multiplexing-compatible CRISPR–Cas9 plasmid bearing the desired sgRNA from Step 19 with FastDigest Eco147I (StuI). Ideally, use a 50-μL reaction system containing 2 μg plasmid DNA, 5 μL 10× FastDigest Buffer, 2 μL FastDigest Eco147I, and 1 μL FastAP thermosensitive alkaline phosphatase (1 U/μL). Incubate at 37 °C for 30 min.

2 Analyze 2 μL of the reaction (add 1 μL of 6× DNA gel loading dye and 4 μL of $ddH_2O$) along with the GeneRuler 1-kb DNA ladder on an agarose gel (1% (wt/vol)) with 1× TAE buffer. Run the gel at 100 V for 30 min and visualize the bands using a Gel Doc XR+ Gel Documentation System.

3 Clean up the gel-confirmed linearized plasmid using the PCR cleanup protocol with a NucleoSpin Gel and PCR Clean-up Kit according to the manufacturer's instructions.

4 Measure the concentration using a NanoDrop 2000 spectrophotometer. Optionally, a gel purification step can also be used for extraction of the linearized plasmid; however, it will typically give a much lower yield.

■ **PAUSE POINT** The linearized plasmids can be stored at −20 °C for up to 3 months.

5 Insert the two (three, if the application is in-frame insertion) fragments obtained from Step 8C(iv) into the StuI-linearized CRISPR–Cas9 plasmid (from Step 3 of this box by Gibson assembly, using NEBuilder HiFi DNA Assembly Master Mix. The required volume of each fragment is calculated on the basis of the spectrophotometer-measured concentrations and a recommended molar ratio of 3:1 (insert/vector). Use a 10-μL reaction system with 100 ng of the linearized plasmid for this Gibson assembly reaction.

▲ **CRITICAL STEP** This equation is used for picomole–nanogram conversion: Picomoles = (weight in nanograms) × 1,000/(base pairs × 650 Da)

6 Flick the tube 3–5 times with a fingertip, spin down the reaction using a Mini Centrifuge (~3,200*g*) at room temperature for 5 s and incubate at 50 °C in a thermocycler for 60 min.

■ **PAUSE POINT** The Gibson reaction can be stored at −20 °C for up to 3 months.

7 Transform 50 μL of One Shot Mach1 T1 phage-resistant competent *E. coli* cells with the complete 10 μL reaction from the above step, using the 42 °C heat-shock transformation protocol as described in Step 13B(ii).

8 Screen the positive clones by *E. coli* colony PCR (see Steps 14–17) with a primer set of StuI-F and StuI-R (see Reagents), which flank the editing template.

▲ **CRITICAL STEP** Amplification of a >2-kb high-GC DNA fragment is relatively challenging. Therefore, we recommend using a custom primer set flanking a ~500-bp region of the joint site of the upstream and downstream homologous recombination templates. An example is shown in Fig. 5d; the primer sequences used (inter1_F and inter1_R) can be found in the 'Reagents' section.

9 Confirm the positive clones (identified with colony PCR) by Sanger sequencing as described in Step 19. Typically, both StuI-F and StuI-R and an additional sequencing primer (inter1_F, Fig. 5d) that can bridge the reads of StuI-F and StuI-R are used to make sure the in-frame deletion/insertion can be read out.

10 Make a 25% (vol/vol) glycerol stock as described in Step 20.

(v) Measure the concentration of each purified fragment using a NanoDrop 2000 spectrophotometer. See Box 2 for details of how to insert the editing templates into CRISPR plasmids.

■ **PAUSE POINT** The purified fragments can be stored at −20 °C for up to 3 months.

9 Linearize the CRISPR plasmids by restriction enzyme digestion. Follow option A if non-multiplexing-compatible CRISPR plasmids are to be constructed and option B for construction of multiplexing-compatible CRISPR plasmids.

▲ **CRITICAL STEP** Because Thermo Fisher Scientific provides a compatible buffer system, both FastDigest restriction enzymes and FastAP thermosensitive alkaline phosphatase can be added at the same time for a total reaction time of 30 min. Instead of a 20-μL digestion, one can enlarge the volume to yield more linearized plasmid that can be stored at −20 °C for up to 3 months for future use.

(A) **Digesting non-multiplexing-compatible CRISPR plasmids**

(i) Prepare a digestion of the selected CRISPR plasmid (pCRISPR–Cas9; pCRISPR–Cas9–ScaligD; pCRISPR–dCas9; pCRISPR–cBEST; or pCRISPR–aBEST, see Fig. 4) for single-editing applications with FastDigest NcoI in a 20-μL digestion reaction containing the following components:

| Component | Amount (μL) | Final concentration |
|---|---|---|
| Plasmid DNA | 10 | 40 ng/μL |
| FastDigest NcoI | 1 | |
| 10× FastDigest buffer | 2 | 1× |
| ddH2O | 7 | |
| Total volume | 20 | |

(ii) Ideally, digest 800 ng of plasmid DNA. Incubate at 37 °C for 30 min. Then add 1 μL of FastAP thermosensitive alkaline phosphatase to the reaction and incubate for additional 10 min at 37 °C.

■ **PAUSE POINT** The linearized plasmids can be stored at −20 °C for up to 3 months.

(B) **Digesting multiplexing-compatible CRISPR plasmids**

(i) Use NcoI and NheI to digest the multiplexing-compatible CRISPR plasmids (Fig. 3). Prepare a double digestion of the multiplexing-compatible CRISPR plasmid (pCRISPR–McBEST) with FastDigest NcoI and FastDigest NheI in a 20-μL digestion reaction containing the following components:

| Component | Amount (μL) | Final concentration |
|---|---|---|
| Plasmid DNA | 10 | 40 ng/μL |
| FastDigest NcoI | 1 | |
| FastDigest NheI | 1 | |
| 10× FastDigest buffer | 2 | 1× |
| $ddH_2O$ | 6 | |
| Total volume | 20 | |

(ii) Ideally, digest 800 ng of plasmid DNA. Incubate it at 37 °C for 30 min and then add 1 μL of FastAP thermosensitive alkaline phosphatase to the reaction and incubate for an additional 10 min at 37 °C, followed by an inactivation step at 75 °C for 10 min.

■ **PAUSE POINT** The linearized plasmids can be stored at −20 °C for up to 3 months.

10 Analyze 2 μL of the above digestion reaction (add 1 μL of 6× DNA gel loading dye and 4 μL of $ddH_2O$) along with the GeneRuler 1-kb DNA ladder on an agarose gel (1% (wt/vol)) with 1× TAE buffer. Run the gel at 100 V for 30 min and visualize the bands using a Gel Doc XR+ Gel Documentation System.

11 Clean up the gel-confirmed linearized plasmid with a NucleoSpin Gel and PCR Clean-up Kit according to the manufacturer's instructions.

▲ **CRITICAL STEP** Optionally, a gel purification step can also be used for extraction of the linearized plasmid; however, it will typically give a much lower yield. The gel purification step can be carried out with a NucleoSpin Gel and PCR Clean-up Kit according to the manufacturer's instructions.

12 Measure the concentration using a NanoDrop 2000 spectrophotometer.

13 To insert the sgRNA cassettes into the digested CRISPR plasmid of choice (and optionally also the editing template, as described in Box 2), follow option A for construction of non-multiplexing-compatible CRISPR plasmids, or option B for construction of multiplexing-compatible CRISPR plasmids.

(A) **Construction of non-multiplexing-compatible CRISPR plasmids**

(i) To clone the ssDNA oligonucleotide containing the 20-nt protospacer from Step 8A(iv) into the linearized single-target CRISPR plasmid using the PCR-free ssDNA oligonucleotide bridging method[18], first prepare a 20-μL reaction mix as follows:

| Component | Amount (μL) | Final concentration |
|---|---|---|
| Linearized plasmid from Step 9A(i) | 1 (30 ng) | 1.5 ng/μL |
| 5 μM oligonucleotide from Step 8A(iv) | 5 | 1.25 μM |
| 2x NEBuilder HiFi DNA Assembly Master Mix | 10 | 1× |
| $ddH_2O$ | 4 | |
| Total volume | 20 | |

(ii) Incubate the reaction for 1 h at 50 °C, using a thermocycler.

▲ **CRITICAL STEP** The reaction volume can be reduced proportionally to 10 μL in order to save reagents.

■ **PAUSE POINT** The reaction can be stored at −20 °C for up to 3 months.

(iii) Transfer 2 μL of the above reaction mixture to 50 μL of in-house-made (or commercial) electroporation-competent Mach1 *E. coli.* cells (see Box 3 for details of making highly efficient electroporation-competent *E. coli*) as follows: remove the 50-μL tubes containing electroporation competent *E. coli* cells from the –80 °C freezer and thaw on ice (~10 min). In the meantime, remove the 1-mm electroporation cuvettes from the −20 °C freezer and place them on ice. Carefully pipette the competent cells into the cuvettes. Mix the competent cells with 2 μL of the ssDNA oligonucleotide bridging reaction from Step 13A(i)

**Box 3 | A modified electroporation-competent cell preparation protocol**

**Procedure ● Timing 2 d**

▲ **CRITICAL** All reagents and equipment that will directly contact the *E. coli* cells need to be pre-sterilized by autoclaving at 121 °C for 20 min.

**Day 1**

1 Place all required reagents and equipment (the centrifuge rotor, 2 L $ddH_2O$, 100 mL 10% (vol/vol) glycerol, 500 mL LB medium, four 250-mL centrifuge bottles, four 50-mL Falcon tubes, and 50 1.5-mL Eppendorf tubes) in a 4 °C cold room for overnight cooling.

2 Inoculate a fresh single colony of the required *E. coli* strain into a 250-mL shake flask containing 50 mL LB medium and incubate at 37 °C with 200 r.p.m. shaking overnight.

**Day 2**

3 Inoculate 5 mL of the overnight culture into a 2-L shake flask with 500 mL LB medium. Incubate the flask at 37 °C with 200 r.p.m. shaking. Measure $OD_{600}$ every hour and every 10 min once the $OD_{600}$ reaches 0.2. After the inoculation, turn on the floor centrifuge and set up a 4 °C mode with the pre-chilled centrifuge rotor.
▲ **CRITICAL STEP** Ensure that the centrifuge is properly cooled to 4 °C. We recommend starting to cool the centrifuge ~1 h in advance.

4 Once an $OD_{600}$ of ~0.4 is observed, bury the whole shake flask (the part with the culture) in ice to chill for 30 min with occasional swirling. Also bury the four 50-mL Falcon tubes, four 250-mL centrifuge bottles, the $ddH_2O$, and the 10% glycerol in ice during this step.
▲ **CRITICAL STEP** It is important not to let the OD value get any higher than 0.4.

5 Subsequently divide the culture equally into the four centrifuge bottles and then harvest the cells by centrifugation at 1,000*g* for 20 min at 4 °C.
▲ **CRITICAL STEP** If conditions permit, carry out the following operations in the 4 °C cold room. Otherwise, perform all operations as quickly as possible and transport *E. coli*-containing bottles/tubes on ice.

6 Carefully decant the supernatant without disturbing the cell pellets and gently resuspend each pellet in 100 mL of ice-cold $ddH_2O$. Combine two resuspensions and then centrifuge again as described in step 5 of this box.
▲ **CRITICAL STEP** Because the cells are becoming more and more competent and fragile, no pipetting should be done to mix or suspend the cells. The time that the cell pellets spend away from the ice needs to be as short as possible.

7 During the centrifugation time, rinse each of the 50-mL Falcon tubes using 10 mL ice-cold 10% (vol/vol) glycerol. Carefully decant the supernatant without disturbing the cell pellets, and gently resuspend each pellet in 40 mL of ice-cold 10% (vol/vol) glycerol. Then transfer the resuspensions to the 50-mL Falcon tubes and centrifuge again as described in step 5 of this box.

8 Repeat Step 7 to wash the cell pellets once more with ice-cold 10% (vol/vol) glycerol to make sure the competent cells are ion free.

9 During the centrifugation time, place the required number of pre-chilled Eppendorf tubes on ice. Prepare liquid nitrogen at this stage as well. Carefully remove and discard the supernatants and then resuspend each pellet in 1 mL of ice-cold 10% (vol/vol) glycerol by carefully swirling. Divide 50 µL of the resuspensions into aliquots in ice-cold 1.5-mL microfuge tubes and snap-freeze them in liquid nitrogen. Then store the electrocompetent cells at −80 °C.
! **CAUTION** Liquid nitrogen is an ultra-low-temperature reagent. Wear safety glasses or a face shield when transferring and operating with liquid nitrogen.
■ **PAUSE POINT** The electrocompetent cells can be stored at −80 °C for up to 6 months.

by flicking the tubes with a fingertip 3–5 times (avoid bubble formation). Use the Ec1 program of a Bio-Rad MicroPulser (alternatively, a similar electroporation program of 1.8 kV with a 1-mm electroporation cuvette with a one-time pulse can be used). Immediately add 200 µL SOC broth to each cuvette and transfer the reaction to a sterilized 1.5-mL Eppendorf tube. Incubate the tube in a heating block at 37 °C with shaking at 800 r.p.m. for 1 h.
▲ **CRITICAL STEP** Chemically competent cells could also be used in this step with the 42 °C heat-shock protocol from Step 13B(ii)

(iv) Plate 100 µL of the reaction onto a selective LB plate supplemented with 50 µg/mL apramycin. Incubate the plate overnight at 37 °C.
! **CAUTION** The transformation efficiency may differ with home-made competent cells. Therefore, we recommend using commercial products if they are available.

(B) **Insertion of the sgRNA cassettes into the multiplexing-compatible CRISPR plasmids by a two-step Golden Gate assembly**

(i) Set up a 10-µL ligation reaction using T4 ligase (5 U), 100 ng of predigested plasmids from Step 9B(i), and the required volume of the prepared sgRNA fragments from Step 8B(vii) to give a 5:1 molar ratio of insert/vector (use the heat-inactivated digestion reaction). Supplement the reaction with 2 µL of 50% (wt/vol) PEG-4000 (included in the T4 ligase kit) and incubate for 1 h at 22 °C.
■ **PAUSE POINT** The reaction can be stored at −20 °C for up to 3 months.
? **TROUBLESHOOTING**

(ii) Transfer 5 µL of the above reaction to 50 µL One Shot Mach1 T1 phage-resistant chemically competent *E. coli*, using a heat-shock protocol as follows: remove 50-µL aliquots of the chemically competent *E. coli* cells from the −80 °C freezer and thaw on ice

(~10 min). Mix with 5 μL of the reaction from Step 13B(i) by flicking with a fingertip (3–5 times). Incubate on ice for 20 min, followed by a 60-s heat shock at 42 °C, using a water bath; steadily transfer the tubes into the ice and incubate for another 5 min. Add 200 μL SOC to the tubes, incubate in a heating block under conditions of 37 °C with 800 r.p.m. shaking for 1 h.

▲ **CRITICAL STEP** Electroporation-competent cells could also be used in this step with an electroporation transformation protocol as described in Step 13A(ii).

(iii) Plate all of the reaction on a selective LB plate supplemented with 50 μg/mL apramycin. Incubate the plate overnight at 37 °C.

14 Screen the clones using an *E. coli* colony PCR as follows: in the morning following Step 13, use sterilized wooden toothpicks to pick 12–24 *E. coli* colonies from each assembly into a 96-deep-well plate containing 300 μL LB medium supplemented with 50 μg/mL apramycin in each well.

15 Incubate the 96-well deep-well plate at 37 °C with 300 r.p.m. shaking for 2 h.

16 Directly use 1 μL of the culture as template DNA for colony PCR with the following setup and conditions.

| Component | Amount (μL) | Final concentration |
|---|---|---|
| sgRNA-TEST-F | 0.5 | 500 nM |
| sgRNA-TEST-R | 0.5 | 500 nM |
| Template DNA | 1 | |
| OneTaq 2× Master Mix with standard buffer | 10 | 1× |
| $ddH_2O$ | 8 | |
| Total | 20 (one reaction) | |

| Cycle no. | Denature | Anneal | Extend | Final |
|---|---|---|---|---|
| 1 | 94 °C, 3 min | | | |
| 2–31 | 94 °C, 30 s | 50 °C, 30 s | 68 °C, 30 s | |
| 32 | | | 68 °C, 5 min | |
| 33 | | | | 10 °C, hold |

17 Analyze 5 μL of the above PCR reaction (add 1 μL of 6× DNA gel loading dye) along with the GeneRuler 1-kb DNA ladder on a long (10-cm) agarose gel (3% (wt/vol)) with 1× TAE buffer. Run the gel at 100 V for 60 min and visualize the bands using a Gel Doc XR+ Gel Documentation System.

▲ **CRITICAL STEP** Because the size difference between the positive and the control is only 20 bp, it takes a >2% (wt/vol) agarose gel with a 60-min run time to distinguish the bands. We recommend using a 10-cm 3% (wt/vol) agarose gel and running the gel at 100 V for 60 min.

■ **PAUSE POINT** The *E. coli* culture in the deep-well 96-well plate can be stored at 4 °C for up to 1 week.

18 Prepare cultures of the above-obtained positive colonies in cultivation tubes containing 5 mL of LB medium supplemented with 50 μg/mL apramycin. Inoculate 50 μL of culture directly from the deep-well 96-well plate and incubate at 37 °C with 200 r.p.m. shaking for ~16 h (overnight).

▲ **CRITICAL STEP** We always use LB medium for cultivating ET12567 *E. coli* strains for conjugation but have observed that 2× YT broth performs better than LB medium for plasmid isolation purposes in general. Therefore, we recommend using 5 mL of 2× YT broth for cultivation of each strain that is going to be used for plasmid isolation.

19 Perform plasmid isolation the following day, using the NucleoSpin Plasmid EasyPure Kit and following the manufacturer's instructions, and submit the plasmids for Sanger sequencing using the sgRNA-TEST-F primer and Cas9-C-terminal-TEST primer. Follow Box 2 to additionally insert the editing template (Fig. 5) after sequencing, if required.

▲ **CRITICAL STEP** We have observed that instability of the pSG5 replicon–based shuttle plasmid in *E. coli* is easily triggered by unknown factors. Therefore, it is critical to confirm the integrity of the CRISPR plasmids. We have observed several cases in which the 'hot region of instability' lies downstream of the *tipA-fd* fragment. Therefore, we recommend running an additional Sanger sequencing with the sequencing primer Cas9-C-terminal. Alternatively, a NdeI–BglII double-digestion mapping can also indicate the integrity of the plasmids.

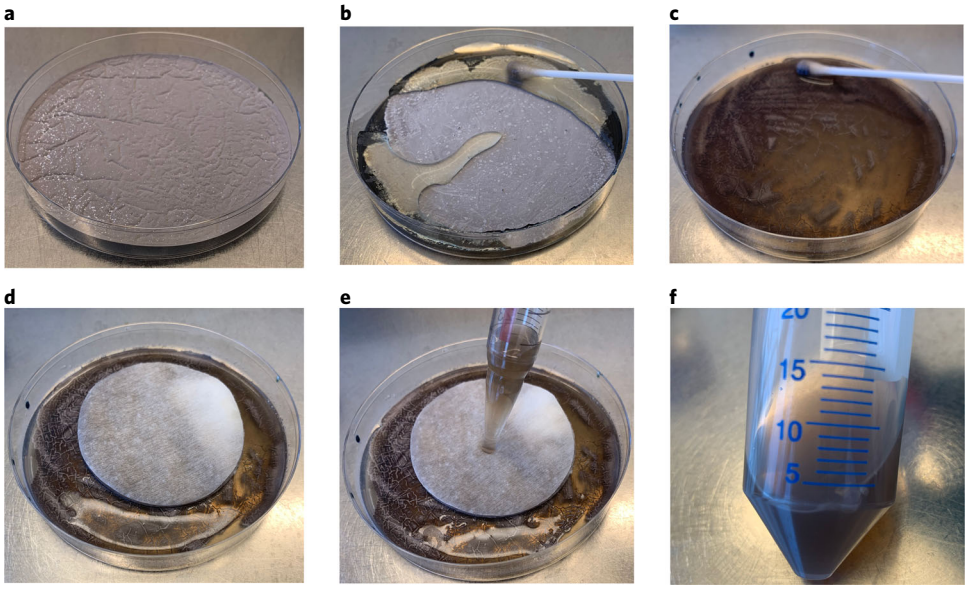


**Fig. 6 | Experimental setup for collecting spores.** A more detailed operation can be found in Supplementary Video 1. **a**, A well-sporulated *S. coelicolor* plate. **b**, Carefully pour 10 mL 2× YT on the spore lawn. **c**, Use a sterilized cotton swab to scrape off the spores. **d**, Carefully place a sterilized cotton pad above the spore suspension with sterilized tweezers. **e**, Use an Integra Pipetboy to aspirate the spore suspension through the cotton pad to remove agar and mycelial debris. **f**, Transfer the spore suspension to a 50-mL Falcon tube.

20 Freeze the *E. coli* strains with the correct plasmids (ready-to-use CRISPR plasmids) confirmed by Sanger sequencing in 25% (vol/vol) glycerol at −80 °C for long-term storage.
**PAUSE POINT** The *E. coli* glycerol stock can be stored at −80 °C for at least 5 years.

### Transfer of ready-to-use CRISPR plasmids into the target streptomycetes by interspecies conjugation ● Timing 2 d

21 Plate the target streptomycete strains onto MS plates for sporulation. This is typically done days ahead, depending on the growth rate; for example, it requires ~5 d for the *S. coelicolor* WT strain to fully sporulate on MS plates at 30 °C.

22 Transfer 200 ng of the desired plasmids from Step 19 into 50 μL of in-house-made electroporation-competent *E. coli* ET (ref. [44]) cells, using the electroporation protocol described in Step 13A(ii) with small modifications as follows: instead of plating onto selective LB plates, here we transfer all the reaction to a 50-mL Falcon tube with 20 mL selective LB medium supplemented with 50 μg/mL apramycin, 25 μg/mL kanamycin, and 12.5 μg/mL chloramphenicol. Incubate the tubes overnight at 37 °C with 200 r.p.m. shaking.
**▲CRITICAL STEP** It is also possible to plate the transformation reaction onto a selective LB plate and then start the culture from a single colony the next day. We recommend using the procedures we provide based on the experience that the DNA methylation–defective *E. coli* ET strain increases the risk of plasmid instability; using a pool of transformants will lower the chance of picking a wrong clone and can save at least 1 d.

23 The following morning, prepare the ET cultures harboring the plasmids of interest, by washing twice using the same volume (20 mL) of antibiotic-free LB medium, and harvest the cells by centrifuging at 5,000*g* for 5 min at room temperature. Then resuspend the cell pellets in 2 mL antibiotic-free LB medium per 20-mL culture.
**▲CRITICAL STEP** We recommend using an amount of LB that is 10% (vol/vol) of the initial culture volume for resuspension of the ET cell pellets.

24 In the meantime, start collecting spores of the streptomycete of interest from Step 21 by carefully pipetting 10 mL 2× YT onto the surface of the spore lawn of a well-sporulated MS plate (Fig. 6a and Supplementary Video 1).

25 Use a sterilized cotton swab to scrape off the spores (Fig. 6b,c and Supplementary Video 1).

26 Carefully place a sterilized cotton pad above the spore suspension with sterilized tweezers (Fig. 6d and Supplementary Video 1).

27 Use an Integra Pipetboy to aspirate the spore suspension through the cotton pad to remove agar and mycelial debris (Fig. 6e and Supplementary Video 1); then transfer the suspension to a 50-mL Falcon tube (Fig. 6f and Supplementary Video 1).
▲ CRITICAL STEP Repeat the above operation twice to maximize the amount of spores from one plate; for strains that produce only low numbers of spores, it may be necessary to collect spores from more than one plate.

28 Collect the spores by centrifuging at 5,000*g* for 5 min at room temperature. Discard the supernatant carefully, resuspend the spores in 2× YT (we typically use 2 mL per plate).

29 Heat-shock the spore suspension for 10 min at 50 °C. The spore suspension now is ready for conjugation.
▲ CRITICAL STEP To achieve higher conjugation efficiency, we recommend leaving the spore suspension at 4 °C overnight (can be up to 3 d) for pre-germination.
■ PAUSE POINT The spore suspension can be used for up to 2 weeks if stored at 4 °C.

30 Mix 500 μL ET suspension from Step 23 with 200 μL spore suspension in a sterilized 1.5-mL Eppendorf tube by pipetting (Supplementary Video 1).

31 Plate 100 μL, 200 μL, and 400 μL of the 700-μL mixture from Step 30 onto three different MS plates, air-dry them in a laminar flow hood for 5 min, and incubate the conjugation plates at 30 °C for ~24 h (overnight).

32 Overlay the conjugation plates with 1 mL sterilized $H_2O$ containing 1 mg apramycin and 1 mg nalidixic acid. Air-dry the plates in a laminar flow hood for 15 min.
! CAUTION We do not recommend using a spreader for the overlay procedure. Instead, try to spread the 1 mL sterilized $H_2O$ containing 1 mg apramycin and 1 mg nalidixic acid just by moving the plate. The 1 mL sterilized $H_2O$ will form clouds after you add the nalidixic acid stock.

33 Incubate the plates until exconjugants can be picked with a sterilized wooden toothpick (an example of ready-to-pick exconjugants is shown in Fig. 7c). Typically, it takes 3–5 d if the streptomycete of interest has a normal growth rate. Transfer the picked exconjugants to a fresh ISP2 plate supplemented with 50 μg/mL apramycin and 50 μg/mL nalidixic acid and incubate for 3–5 d at 30 °C.
▲ CRITICAL STEP Almost all currently available genetic manipulation methods for streptomycetes require that the streptomycete strains be able to take up the plasmids. This makes conjugation a critical step of genome editing. The efficiency of conjugation can be affected by many factors. Our protocols have been successfully used for various actinomycete strains[9,15,32–38].
? TROUBLESHOOTING

## Evaluation of the successfully edited strains ● Timing 5–14 d

34 Validate the mutants. Use option A for evaluation of non–genetically edited applications (CRISPRi); follow option B for chromosomally edited applications or option C to further evaluate genetically edited mutants by Illumina sequencing.

(A) **Evaluation of non–genetically edited applications**

(i) Prepare seed cultures by inoculating three randomly picked exconjugants (from Step 33) for each plasmid and a nontreated control into individual shake flasks, each containing 50 mL selective ISP2 broth, and incubate at 30 °C, with 180 r.p.m. shaking for 3 d.

(ii) To normalize the start amount for the above seed cultures by wet weight, spin down 1-mL cultures at 10,000*g* at room temperature for 5 min. This is to enable measurement of equal amounts of inocula for the main cultures as described in the next step.

(iii) Inoculate 500-mg cell pellets (wet weight) from seed cultures of each exconjugant from Step 34A(ii) into a fresh 50-mL selective ISP2 broth–containing shake flask and incubate the flask at 30 °C with 180 r.p.m. shaking for 3–5 d.

(iv) Extract and analyze the endpoint product accordingly. For example, actinorhodin, the endpoint product of the actinorhodin biosynthetic pathway, can be analyzed by absorbance measurement. A detailed example can be found in ref. [9].
! CAUTION Besides directly analyzing the endpoint product[9], transcription analysis can also be applied, for example, qRT-PCR and RNA-seq.
? TROUBLESHOOTING

(B) **Evaluation of genetically edited strains by colony PCR**

(i) Quick screening of the correctly edited clones can be achieved by following a *Streptomyces* colony PCR protocol. First, use a sterile wooden toothpick to scratch ~4 $mm^2$ of mycelia

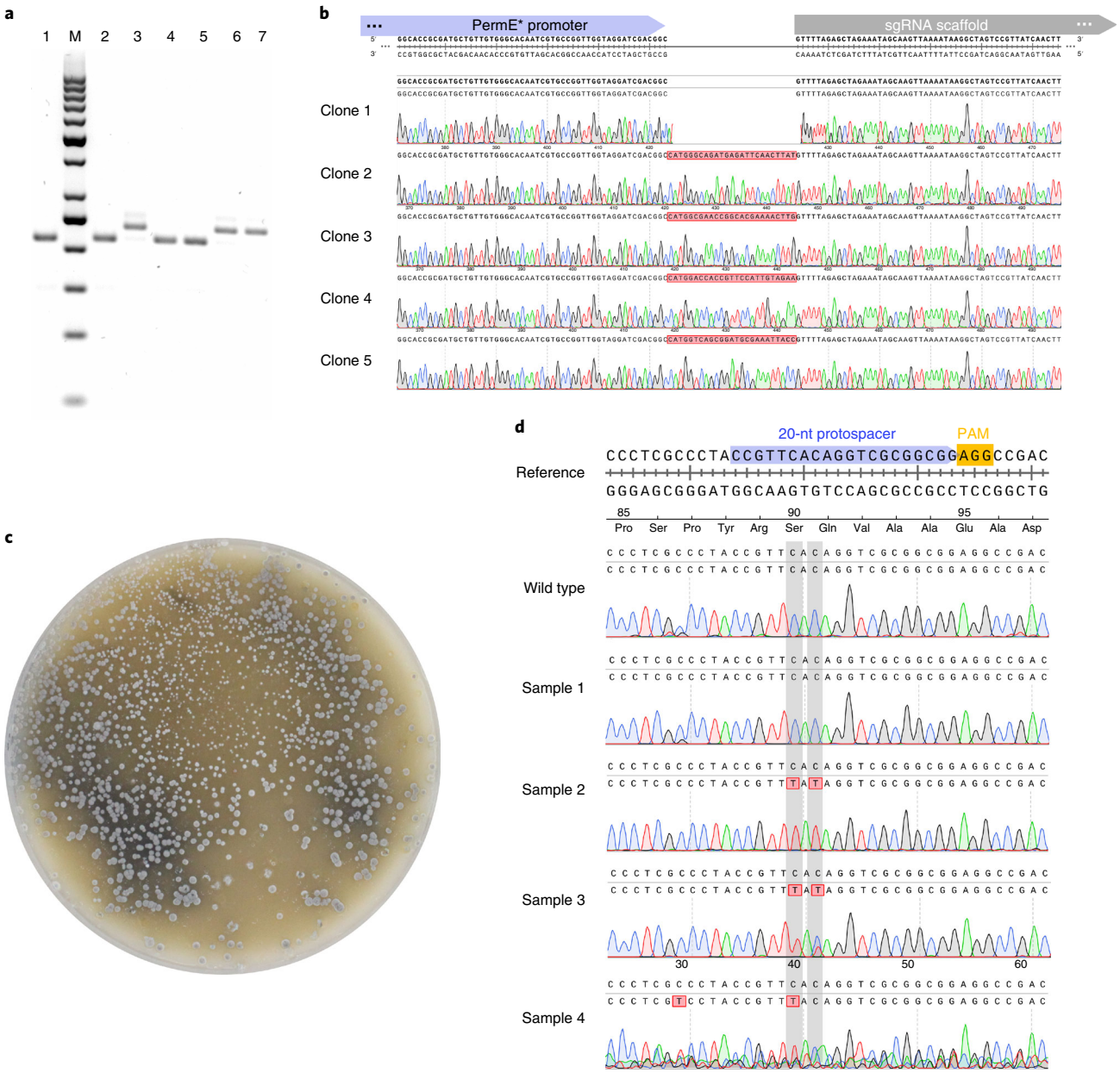


**Fig. 7 | Anticipated results. a**, Colony PCR screening of the 20-bp spacer cloning; the results are visualized by a 3% (wt/vol) agarose gel. Lane 1: negative control without the 20-bp spacer; lanes 3, 6, and 7: clones with 20-bp spacer inserted; lanes 2, 4, and 5: clones without 20-bp spacer inserted; lane M: GeneRuler 50-bp DNA Ladder, 13 bands (top to bottom) represent DNA sizes of 1,000, 900, 800, 700, 600, 500, 400, 300, 250, 200, 150, 100, and 50 bp, respectively. **b**, PCR screening and confirmation by Sanger sequencing of potentially positive clones. Clone 1 is a negative control without inserted spacer, whereas Clones 2–5 represent positive clones with a 20-bp; the different 20-bp spacers are highlighted in red boxes. **c**, Photograph of a successful conjugation experiment with >50 pickable colonies. **d**, Examples of the possible Sanger sequencing output of a base-editing application targeting the *SCO5087* gene of *S. coelicolor* using pCRISPR-cBEST. The 20-nt protospacer sequence is highlighted in blue, whereas the 3-nt PAM sequence is shown in yellow. The two target cytosines and their expected edits are highlighted in gray. The variant nucleotides (as compared to the reference sequence) are highlighted in red.

from the ISP2 plates from Step 33 (after incubation for 3–5 d) and transfer the mycelia to a PCR tube containing 20 μL DMSO.

▲ **CRITICAL STEP** We recommend using the mycelia from a fast-growing stage (before you can see the sporulation) for *Streptomyces* colony PCR.

▲ **CRITICAL STEP** Because most of the streptomycetes are mycelial-growing bacteria, they have certain growth stages during which there are multiple chromosomes in one cell. This can sometimes lead to a mixed population of WT and edited cells. Taking

into consideration that different sgRNAs often have different editing efficiencies, we recommend applying a single-spore separation to the re-streaked colonies from Step 33. A simple single-spore isolation can be done by touching the sporulated colony with a sterilized wooden toothpick, dipping the toothpick into 200 µL sterilized $ddH_2O$, and then plating 20, 50, and 100 µL of the above-obtained spore suspension onto three induction ISP2 plates. After 3–5 d of incubation at 30 °C, single colonies are expected to appear on the plates.

(ii) Incubate for 15 min at 100 °C, with shaking at 1,000 r.p.m. using a shaking/heating block.

(iii) Transfer the tube to a −20 °C freezer and freeze for 30 min.

▲ **CRITICAL STEP** Breaking up streptomycete cells can be improved by repeating the 100 °C−20 °C cycle one more time.

(iv) Use 1.5 µL of the obtained solution as template DNA for colony PCR. Primers flanking the ~500-bp target regions are used, for example inter1_F and inter1_R (see 'Reagents' section and Fig. 5a). Perform PCR as follows:

| Component | Amount (µL) | Final concentration |
|---|---|---|
| Forward primer | 1 | 400 nM |
| Reverse primer | 1 | 400 nM |
| Template DNA | 1.5 | 3% (vol/vol) DMSO |
| 2× Phusion High-Fidelity PCR Master Mix with HF Buffer | 25 | 1× |
| $ddH_2O$ | 21.5 | |
| Total | 50 (one reaction) | |

| Cycle no. | Denature | Anneal | Extend | Final |
|---|---|---|---|---|
| 1 | 98 °C, 1 min | | | |
| 2–31 | 98 °C, 10 s | 65 °C[a], 30 s | 72 °C, 10 s | |
| 32 | | | 72 °C, 5 min | |
| 33 | | | | 10 °C, hold |

[a]Annealing temperature is from $T_m$ calculator.

▲ **CRITICAL STEP** We obtained equal efficiencies when using NEB Q5 High-Fidelity 2× Master Mix and 2× Phusion High-Fidelity PCR Master Mix with HF Buffer.

(v) Analyze 5 µL of the above PCR reaction (add 1 µL of 6× DNA gel loading dye) along with the GeneRuler 1-kb DNA ladder on an agarose gel (1% (wt/vol)) with 1× TAE buffer. Run the gel at 100 V for 30 min and visualize the bands using a Gel Doc XR+ Gel Documentation System.

(vi) Clean up the above PCR products with clear bands using a NucleoSpin Gel and PCR Clean-up Kit and following the manufacturer's instructions. Measure the concentration of each fragment using a NanoDrop 2000 spectrophotometer.

(vii) Validate the edits by Sanger sequencing of 8–12 PCR products, using the forward primers flanking the target regions.

**? TROUBLESHOOTING**

(C) **(Optional but highly recommended) Evaluation of genetically edited mutants by Illumina sequencing**

(i) Inoculate a single colony each of the Sanger sequencing–validated positive strain and a WT parental strain into independent 50-mL shake flasks containing ISP2 broth (with no antibiotics) and incubate at 30 °C with 180 r.p.m. shaking for 3–5 d.

(ii) Isolate genomic DNA from the above strains using the Qiagen Blood & Cell Culture Midi DNA Kit with a modified protocol shown in Box 4.

■ **PAUSE POINT** The genomic DNA solution can be stored at −20 °C for up to 6 months.

(iii) Build the Illumina sequencing libraries using a KAPA HyperPlus Kit, aiming for an insert size of ~600 nt, using SeraMag Select SPRI beads and following the manufacturer's instructions.

**Box 4 | Modified protocol for using the Qiagen Blood & Cell Culture Midi DNA Kit with streptomycetes ● Timing 0.5–1 d**

**Procedure**

1 Harvest the cell pellets from 10 mL of the 5-d-old streptomycete-of-interest culture by centrifugation at 10,000*g* for 10 min at room temperature.
2 Discard the supernatant and resuspend the above pellets in 3.5 mL Buffer B1 with 70 μL RNase A solution (10 mg/ml) by vortexing at top speed for 10 s.
3 Add 100 μL lysozyme stock solution (100 mg/mL) and 100 μL protease K solution, and then incubate in a water bath at 37 °C for 60 min.
**! CAUTION** The incubation time may need to be prolonged if a clear lysis is not observed. Overnight incubation at 4 °C is acceptable.
4 Add 1.2 mL Buffer B2, mix it well by inverting the tube 5–10 times, and incubate the tube in a water bath at 50 °C for 60 min.
5 During the last 10 min of the incubation, equilibrate a Qiagen Genomic-tip 100/G (from the kit) with 4 mL Buffer QBT from the Blood & Cell Culture DNA Mini Kit. Empty the Qiagen Genomic-tip by gravity flow.
6 Vortex the lysis sample from step 4 of this box for 10 s at top speed and load it to the equilibrated Qiagen Genomic-tip. Allow it to pass by the resin by gravity flow.
7 Wash the Qiagen Genomic-tip with 2 × 7.5 mL Buffer QC.
8 Elute the genomic DNA with 5 mL Buffer QF into a 15-mL Falcon tube.
9 Precipitate the DNA by adding 3.5 mL room-temperature isopropanol and invert the tube 5–10 times.
10 Centrifuge the above solution immediately at 10,000*g* for 20 min at 4 °C. Carefully remove and discard the supernatant by pipetting.
11 Wash the DNA pellets with 2 mL ice-cold 70% (vol/vol) ethanol twice.
12 Carefully remove the last droplets by pipetting, and air-dry the DNA pellets for 5 min at room temperature.
13 Dissolve the DNA pellets with 100 μL dd$H_2O$ (pH was adjusted to 8.5 using 1 M NaOH) at 55 °C for 1 h.
14 Run 1 μL of the above PCR reactions and GeneRuler 1-kb DNA ladder on an agarose gel (0.7% (wt/vol)) with 1× TAE. Run the gel at 100 V for 30 min and visualize the bands using a Gel Doc XR+ Gel Documentation System to check the integrity of the isolated genomic DNA.
15 Measure the 260/280 and 260/230 absorbance ratios using a NanoDrop 2000 spectrophotometer, and measure the concentration with a Qubit 2.0 Fluorometer.
**! CAUTION** A good genomic DNA sample for Illumina sequencing should have the 260/280 and 260/230 ratios at ~1.8 and ~2.2, respectively.
**? TROUBLESHOOTING**

**▲ CRITICAL STEP** Using a kit with few PCR cycles is preferable because it avoids a high proportion of clonal fragments. Similarly, because *Streptomyces* has a high GC content, a transposase-based sequencing library kit is highly discouraged because the fragment diversity would be low.

(iv) Quantify the libraries by using a fragment analyzer and a Qubit dsDNA HS Assay Kit and following the manufacturer's instructions.

(v) Sequence the samples on a suitable Illumina machine with a paired-end protocol of 2× 150 nt (or longer), such as a NextSeq500 sequencer, to a coverage of ~100.

(vi) After base-calling the paired-end reads, perform adaptor and quality trimming of the reads, for example, by using AdapterRemoval2 (ref. [47]) with the switches `--trimns` and `--trimqualities`.

(vii) Run FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc) to ensure that low-quality reads and sequences have been sufficiently removed from the trimmed dataset.

(viii) Use breseq[48] (e.g., v.0.33.2) with a high-quality reference genome sequence to estimate the changes in both the WT and genome-edited strains. We suggest running breseq without the switch `--polymorphism-prediction` because local low coverage and other technical noise can lead to false low-frequency off-target predictions. Evaluate the reports of both the WT and genome-edited strains and report the predicted mutations from all datasets. Identical predicted 'mutations' in the two datasets could reflect mutations that happened before the genome editing experiments. Pay special attention to larger structural changes of the streptomycetes genomes, such as deletions in the chromosome ends, which would probably happen in DSB-based genome editing but rarely in DSB-free genome editing, that is, CRISPR-BEST.

**! CAUTION** The WT strain from the same origin can accumulate different spontaneous mutations when it is maintained and used in the laboratory over time. Therefore, we highly recommend including the parental strain when evaluating the on/off-target edits from CRISPR genome editing using whole-genome sequencing–based techniques; otherwise, off-target effects are likely to be overestimated.

**? TROUBLESHOOTING**

### (Optional) Plasmid curing ● Timing 7–14 d

▲ CRITICAL For a second round of editing using the same type of pSG5-based CRISPR plasmid, the CRISPR plasmids from the successfully edited streptomycete strains first must be eliminated using the following steps.

35 Inoculate a single colony of the successfully edited streptomycete strain from Step 34B(vii) into a 250-mL shake flask containing 50 mL nonselective ISP2 broth (with 3.4% (wt/vol) sucrose) and incubate at 40 °C with 180 r.p.m. shaking for 3–5 d to reach the exponential growth phase.

! CAUTION The incubation time depends on the target strain itself.

36 Plate an appropriately (1,000- to 10,000-fold, depending on the density of the obtained culture) diluted fraction of the above culture on a nonselective ISP2 plate and incubate it at 30 °C for 3–5 d to obtain single colonies.

▲ CRITICAL STEP We recommend having ~100–200 single colonies on each standard 10-cm Petri dish.

37 Replicate the above plate containing single colonies onto another 50 μg/mL apramycin-supplemented ISP2 plate.

! CAUTION Owing to the vegetative growth feature of *Streptomyces*, it is relatively difficult to replicate colonies from one plate to another. We recommend directly picking and streaking well-marked colonies using sterilized wooden toothpicks.

38 After 3–5 d incubation at 30 °C, some colonies with restored apramycin sensitivity can be observed. These are CRISPR plasmid cured strains.

39 For long-term storage, prepare a 25% (vol/vol) glycerol stock of a spore suspension or liquid culture at the exponential growth phase of the correctly edited strain with the CRISPR plasmids (from Step 34B) or without the CRISPR plasmids (from Step 38) and store at −80 °C.

■ PAUSE POINT The glycerol stock can be stored at −80 °C for at least 5 years.

## Troubleshooting

Troubleshooting advice can be found in Table 1.

**Table 1 | Troubleshooting table**

| Step | Problem | Possible reason | Solution |
|---|---|---|---|
| 1A | CRISPy-web reports 'Invalid input file' on upload | Uploaded file is not a valid GenBank file and/or was corrupted during upload | Verify that the input file is a valid GenBank file and retry the upload |
| 2 | On the overview page, the details box lists '0 genes' | No valid CDS records found in the GenBank file | Add gene annotations using a gene-finding tool such as RAST (https://rast.nmpdr.org/, for an online service) or Prodigal (https://github.com/hyattpd/Prodigal, as a local tool) |
| | On the overview page, no antiSMASH clusters are listed | Used the wrong input file | Check whether you used the antiSMASH download or uploaded an antiSMASH result file |
| | | antiSMASH found no clusters | Select target region using genomic coordinates or locus tags instead |
| 4 | No CRISPR-BEST protospacers shown on gene of interest | No combination of PAM and edit window introducing an amino acid change is present on the gene of interest | Select a different gene to edit |
| 8B(iii) | No bands | Forgot to add primers or DNA template | Double-check the components and rerun the PCR |
| | Unspecific bands | Given the large primer overhangs, especially of the first sgRNA, additional optimization of the reaction may be required | Addition of 3% (vol/vol) DMSO might improve the results. Successful elimination of unspecific bands was further achieved by running a touchdown PCR |
| 8C(iii) | No bands | Forgot to add primers or DNA template | Double-check the components and rerun the PCR |
| | PCR unsuccessful; strong background | A direct colony PCR was used. The strong background of the PCR templates will increase the chances of unspecific amplifications or PCR failure | Reduce the amount of the lysed *Streptomyces* templates<br>Use purified genomic DNA as templates<br>Use a touchdown PCR<br>Use another high-GC-friendly PCR kit |
| 13B(i) | Ligation not successful | Unsuccessful ligation was probably caused by insufficient purity of the fragments and/or backbone, or by incompatible overhangs | It is highly recommended to extract fragments from an agarose gel, unless the amplification was highly specific. Furthermore, after purification, the values of |

Table continued

**Table 1 (continued)**

| Step | Problem | Possible reason | Solution |
|---|---|---|---|
| | | | A260/A280 and A260/A230 should both be >1.8. Incompatible overhangs will also result in no ligation. We recommend double-checking and/or performing in silico cloning to verify that the overhangs produced by BsaI digestion are compatible |
| 33 | Too few exconjugants | Check concentration of spores and/or *E. coli* ET cultures | Increase the amount of both the spores and the ET cultures |
| | Unsuccessful conjugation | Compromised CRISPR plasmid in ET or in the target streptomycete strain due to the plasmid instability | Sanger-sequence the region of the pSG5 replicon of the suspicious plasmid from the ET strain to confirm its integrity |
| | | | A simpler solution can be using the plasmid from another clone and redoing the transformation to ET and the following conjugation |
| | | The pSG5 replicon is not compatible with the target streptomycete strains. | When working with nonstandard streptomycetes, it is advisable to check whether the pSG5 replicon is compatible. If not, the plasmid cannot be used without replacing the pSG5 replicon |
| | | Cross-contamination of *E. coli* ET with another *E. coli* strain | Double-check that the *E. coli* strain used for conjugations is *E. coli* ET. Always use all three antibiotics: kanamycin, chloramphenicol, and apramycin |
| | | Wrong media composition or preparation | Correct media preparation is crucial for successful conjugations. For specific nonstandard streptomycetes, we observed greatly reduced conjugation efficiencies when full-fat soy flour was used. Furthermore, adding $MgCl_2$ before autoclaving can greatly reduce the number of sporulating exconjugants |
| 34A(iv) | No or only little CRISPRi effects are observed | Nonfunctional and/or incorrect protospacers were used | Double-check the protospacer selection; refer to the 'sgRNA design' section for more information |
| | | | Because CRISPRi has a knockdown effect, we recommend testing at least 3 protospacers from different locations |
| | | | Instead of using the yield of the endpoint products for evaluation, try using qRT-PCR or RNA-seq to evaluate the suppression of the transcription |
| | | *tipA* promoter does not work or works with low efficiency in the strain of interest | Replace the *tipA* promoter with an approved functional promoter |
| | | Analysis of the endpoint products was conducted at an inappropriate time | Because CRISPRi can only suppress the transcription, the endpoint products are still accumulating during the entire cultivation time; if this time period is too long, a saturation effect of the endpoint products might mask the production differences between the mutant and the WT. We recommend picking a good time for analysis of the endpoint products or using qRT-PCR or RNA-seq to evaluate the suppression of the transcription |
| 34B(vii) | Mixed sequencing signal (Fig. 7d) | Multiple chromosomes present in the cell; a mixed and onlypartially edited population was obtained | Re-streaking the exconjugant to obtain a single colony, followed by resequencing, is recommended. For reads with mixed signals but <20% of the unwanted signal, in most cases the edited phenotype is persistent. To verify a successful editing outcome, re-streaking and resequencing are recommended |
| | Low editing efficiency | A protospacer with low editing efficiency was used. This could, for example, be caused by sequence properties that allow formation of an alternative RNA structure rather than the correct one | Use an RNA structure prediction software to see whether the used spacer is good |
| | | | In some streptomycetes, induction might be required. Re-streaking exconjugants on ISP2 plates supplemented with apramycin and thiostrepton, followed by resequencing, is recommended |
| | | | A simpler solution can be just repeating the experiment with another re-picked protospacer |

Table continued

**Table 1 (continued)**

| Step | Problem | Possible reason | Solution |
|---|---|---|---|
| | | *tipA* promoter does not work or works with low efficiency in the strain of interest | Replace the *tipA* promoter with an approved functional promoter |
| | | For CRISPR-BEST, the target C or target A might be located in a suboptimal sequence context, which negatively influences the editing efficiency (e.g., if the target C is in a GC context) | Pick another protospacer |
| 34C(viii) | An unexpected large number of off-target effects are observed between the edited strain dataset and the database reference genome | The reference genome does not accurately reflect the parental strain used for genome editing | We suggest using the parental dataset with a polishing tool such as Pilon[50] to change the database reference genome to better reflect the parental strain. Then rerun breseq on both the parental and the edited strains |
| | Unexpected large or important parts of the reference genome are not covered by the Illumina data | A failure probably happened during the Illumina sequencing process | We highly recommend re-purifying the genomic DNA and rebuilding the Illumina library using fewer PCR cycles, because increased numbers of PCR cycles decrease the fragment diversity, particularly in high-GC regions |
| Box 4 | Low or no yield of genomic DNA | Overloaded tip | Reduce the volume by half for cell harvest |
| | | Incomplete lysis reaction | Double the amount of the lysozyme and/or prolong the incubation time |
| | Degraded genomic DNA samples | DNA degradation can be caused by a contamination of DNase and/or too much physical force during preparation | Replace the potentially contaminated reagents and reduce the use of unnecessary physical force |

## Timing

The minimal timing is based on optimal conditions, that is, receiving DNA oligonucleotides from the provider the next day after ordering; receiving Sanger sequencing results from the Sanger sequencing provider the next day after sample submission; using target streptomycete strains such as *S. coelicolor* WT with a relatively fast growth; and having all required reagents, including competent cells, prepared beforehand.

Steps 1–7, sgRNA design: 30 min

Steps 8–20, construction and validation of the desired CRISPR plasmid: 7–16 d

Steps 21–33, transfer of ready-to-use CRISPR plasmids into target streptomycetes by interspecies conjugation: 2 d

Step 34, evaluation of the successfully edited strains: 5–14 d

Steps 35–39, (optional) plasmid curing: 7–14 d

Box 2, inserting editing templates into CRISPR plasmids for in-frame deletion or foreign DNA insertions: 3–5 d

Box 3, a modified electroporation-competent cell preparation protocol: 2 d

Box 4, modified protocol for using the Qiagen Blood & Cell Culture Mini DNA Kit with streptomycetes: 0.5–1 d

## Anticipated results

### sgRNA cloning efficiency

The ssDNA bridging method for 20-nt protospacer cloning generally results in cloning efficiencies of >50% (highly dependent on the sequence of the protospacers). The first step of validation can be colony PCR, and the results can be visualized on a 3–4% (wt/vol) agarose gel (Fig. 7a). The obtained positive clones can then be confirmed by Sanger sequencing (Fig. 7b) as the second step of validation.

### Conjugation efficiency

Using the optimized conjugation protocols described here, we expect to obtain >200 exconjugants per 100 ng of starting plasmid DNA material on one MS plate (Fig. 7c).

### A random-sized deletion library construction of custom size using plasmid pCRISPR–Cas9

Application of this method requires that the target streptomycete strain have a defective NHEJ pathway; that is, the ligase component is not fully functional[9]. Because the essential gene–compromised clones cannot survive, all colonies from the selection plates are either edited or plasmid carrying but non-edited. Considering the high editing efficiency that we observed in *S. coelicolor* A3(2) (ref. [9]), we expect to see >80% of the picked colonies bearing random-sized deletions of non-essential genetic regions[9]. The library size can be controlled by the amount of the starting plasmid. The validation of the library requires whole-genome sequencing.

### Loss-of-function mutation

Within the toolkit described here, three plasmids can be used to inactivate a gene: (i) When using pCRISPR–Cas9–ScaligD, one can expect mutants bearing small indels around the DSB sites; the mutations can be validated by standard Sanger sequencing (80% editing efficiency is expected[9]). (ii) When using pCRISPR–Cas9 with editing templates, one can expect a precise in-frame deletion mutant (with a well-designed editing template, an in-frame insertion mutant can be achieved), which also can be validated by standard Sanger sequencing (95% editing efficiency is expected[9]). (iii) When using pCRISPR–cBEST, one can expect mutants with stop codon introduction; again, the mutations can be validated by standard Sanger sequencing (90% editing efficiency is expected[15]). By visualizing the sequencing trace files in SnapGene or CLC Main Workbench, one can determine whether the editing took place, identify bad sequencing traces due to PCR and/or sequencing processes, identify a mixed population of edited and unedited cells, and confirm the expected clean editing (Fig. 7d).

### Gene transcriptional modulation with CRISPRi (pCRISPR–dCas9)

By using pCRISPR–dCas9, one can expect decreased transcription of the gene of interest[9].

### Accession codes

The plasmids described in the protocols are available at Addgene (pCRISPR–Cas9, 125686; pCRISPR–dCas9, 125687; pCRISPR–Cas9–ScaligD, 125688; pCRISPR–cBEST, 125689; and pCRISPR–aBEST, 131464) or upon request.

### Reporting Summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

### Data availability

No new data were generated or analyzed with this protocol; all presented data were previously published[9,15].

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## Author contributions

Y.T. designed and developed the protocol; K.B. designed the spacer identification software CRISPy-web; T.S.J. established the genome-wide off-target evaluation pipeline. Y.T., and C.M.W. performed the experiments; T.W. and S.Y.L. supervised and steered the project; and Y.T., T.W., and S.Y.L. wrote the protocol.

## Competing interests

Y.T., T.W., and S.Y.L. are co-inventors on a patent application for an actinomycete CRISPR application (WO2016150855A1) filed by Technical University of Denmark.

## Additional information

**Supplementary information** is available for this paper at https://doi.org/10.1038/s41596-020-0339-z.

**Correspondence and requests for materials** should be addressed to T.W. or SY.L.

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**Key references using this protocol**

Tong, Y., Charusanti, P., Zhang, L., Weber, T. & Lee, S. Y. *ACS Synth. Biol.* **4**, 1020–1029 (2015): https://doi.org/10.1021/acssynbio.5b00038

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| Data analysis | SnapGene Version 4.1.8 and CLC Main Workbench Version 8.0.1 were used to display and analyze DNA sequences. Whole genome mapping was carried out by BWA with the BWA-mem algorithm, and then was inspected and visualized using readXplorer and Artemis. |

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