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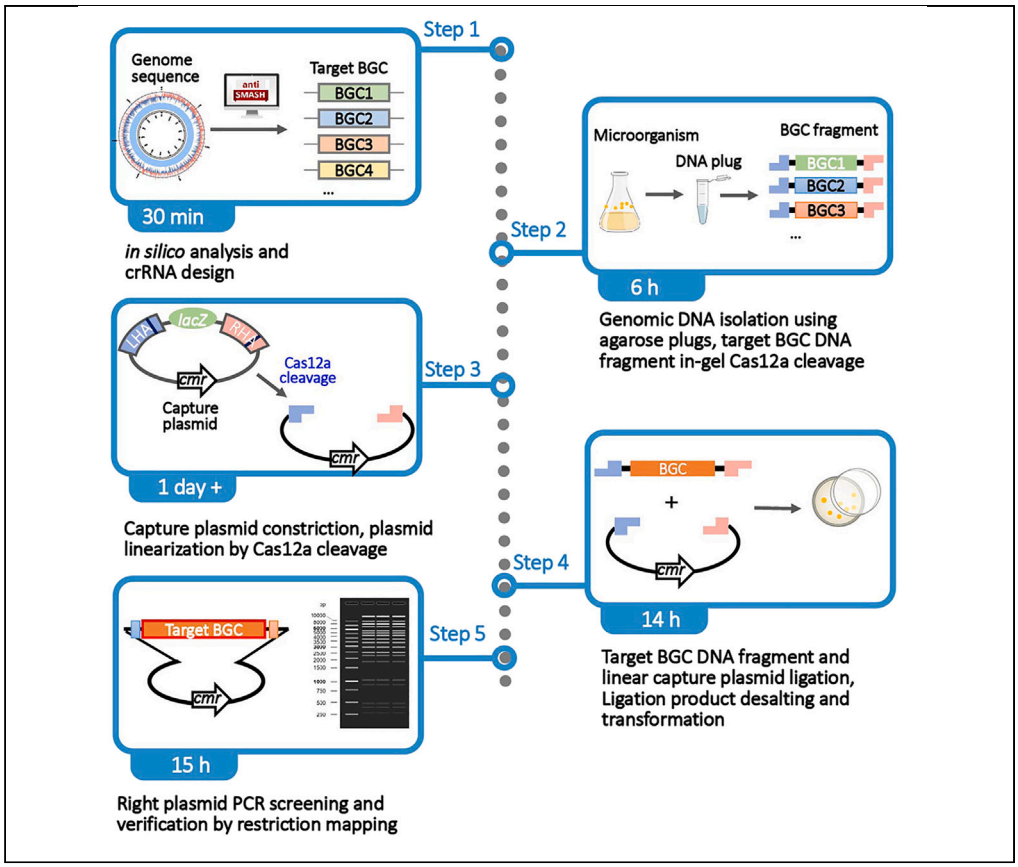
Protocol

An *in vitro* CRISPR-Cas12a-mediated protocol for direct cloning of large DNA fragments



Large biosynthetic gene cluster (BGC) cloning is important for discovering natural product-based drugs and remains challenging in high GC content microorganisms (e.g., Actinobacteria). Here, we present an *in vitro* CRISPR-Cas12a-mediated protocol for direct cloning of large DNA fragments. We describe steps for crRNA design and preparation, genomic DNA isolation, and CRISPR-Cas12a cleavage and capture plasmid construction and linearization. We then detail target BGC and plasmid DNA ligation and transformation and screening for positive clones.

Xiaoqian Zeng, Shuliu Wang, Mindong Liang, ..., Yaojun Tong, Lixin Zhang, Gao-Yi Tan

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Highlights

Steps for combining Cas12a and the advanced features of BAC library construction

Adaptable for cloning from organisms with complicated genomic DNA sequence

Obtain a 145-kb BGC from high GC genomic DNA through *in vitro* direct cloning

Zeng et al., STAR Protocols 4, 102435
September 15, 2023
https://doi.org/10.1016/j.xpro.2023.102435

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An *in vitro* CRISPR-Cas12a-mediated protocol for direct cloning of large DNA fragments

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https://doi.org/10.1016/j.xpro.2023.102435

SUMMARY

Large biosynthetic gene cluster (BGC) cloning is important for discovering natural product-based drugs and remains challenging in high GC content microorganisms (e.g., Actinobacteria). Here, we present an *in vitro* CRISPR-Cas12a-mediated protocol for direct cloning of large DNA fragments. We describe steps for crRNA design and preparation, genomic DNA isolation, and CRISPR-Cas12a cleavage and capture plasmid construction and linearization. We then detail target BGC and plasmid DNA ligation and transformation and screening for positive clones. For complete details on the use and execution of this protocol, please refer to Liang et al.[1]

BEFORE YOU BEGIN

In the post-genome era, the whole genome-sequenced microorganism has revealed a large amount of unexploited cryptic biosynthetic gene clusters (BGCs) which may encode novel bioactive small molecules.[2] Obtaining these BGC DNA fragments is the prerequisite for activating or overproducing them in suitable cell hosts.[3] However, high-efficient cloning of large DNA fragments for example BGCs from microorganisms with complicated genomic DNA (e.g., high G + C content *Actinobacteria*) is still very challenging.

CRISPR/Cas12a is a single RNA-guided (crRNA) endonuclease with a TTTV PAM site.[4] Unlike the blunt end formed by Cas9 protein, cas12a cleaves double stranded DNA to form a 4 or 5 nt sticky end.[5,6] Cas12a has been widely used in genome editing,[7–9] nucleic acid detection[10,11] and small molecule detection *etc.*[12,13] Moreover, Cas12a-based DNA assembly methods, including C-Brick[14] and CCTL,[15] have been developed for assembling exogenous DNA fragments in *E. coli*. These studies suggest that Cas12a-mediated generation of target fragments with sticky ends can lead to simpler and more efficient DNA assembly and plasmid construction.

In this protocol, we describe the steps for obtaining large BGCs from *Streptomyces* in detail by integrating CRISPR/Cas12a and the advanced features of bacterial artificial chromosome (BAC) library construction (designated CAT-FISHING, CRISPR/Cas12a-mediated fast direct biosynthetic gene

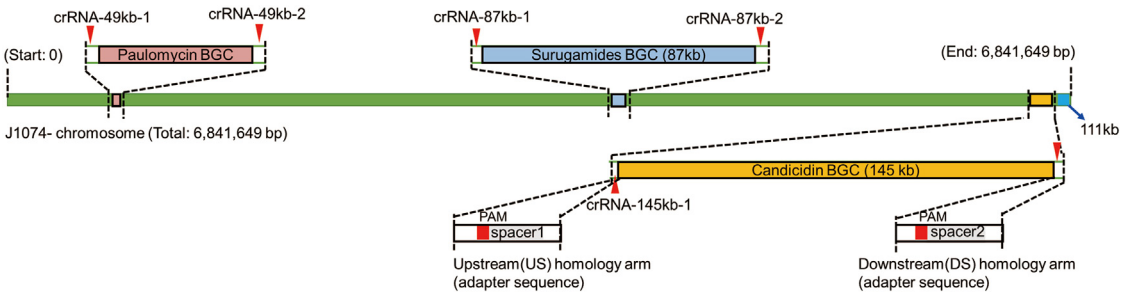


Figure 1. Genomic locations of the three selected BGCs (49, 87 and 145 kb) in the chromosome of *S. albus* J1074

Under the guidance of three crRNA pairs (crRNA-49kb-1/2, crRNA-87kb-1/2, crRNA-145kb-1/2), the three corresponding BGCs could be directly cut from the genomic DNA of *S. albus* J1074 by Cas12a. Next to the 145kb gene cluster, a 111kb DNA sequence at the end of linear chromosome could be released simultaneously. The figure was adapted from Liang et al. (2022).[1]

cluster cloning). Instead of using restriction enzymes (e.g., *HindIII*, *EcoRI*) to partially digest the genomic DNA during BAC library construction,[16,17] this protocol uses Cas12a together with specific crRNA pairs to precisely cut the genomic DNA. Compared to using the Gibson assembly method,[18] Red/ET recombination[19] or Cre-lox mediated *in vivo* recombination[20] for plasmid construction in CATCH (Cas9-assisted targeting of chromosomal segments), ExoCET (Exonuclease Combined with RecET recombination), and CAPTURE (Cas12a-assisted precise targeted cloning using *in vivo* Cre-lox recombination), respectively, CAT-FISHING utilizes DNA ligase to join the target BGC and the BAC plasmid. And CAT-FISHING has been proven to be a fast and efficient strategy for targeting large BGCs (e.g., >120 kb) from high-GC (e.g., >70%) genomic DNA through *in vitro* direct cloning.

As demonstrations, several large BGCs from different actinomycetal genomic DNA samples were efficiently captured by CAT-FISHING, the largest of which was 145 kb with 75% GC content.[1] Although this protocol describes how to directly clone BGC from *Streptomyces* using CAT-FISHING, it also can be used to clone large DNA fragments from other organisms.

In silico analysis of target BGC and crRNA design

Timing: 30 min

This step describes crRNA pairs designed to directly cut the BGC of interest from genomic DNA by CRISPR/Cas12a. In this protocol, three previously reported BGCs (49-kb paulomycin BGC, 87-kb surugamide BGC and 145-kb candicidin BGC; Figure 1) in *Streptomyces albus* J1074 are selected as example.[21]

1. Visualize the target genome sequence or BGC sequence (*S. albus* J1074, GenBank: GCA_000359525.1) by the SnapGene® viewer software (https://www.snapgene.com/).
2. The upstream (US) and downstream (DS) homology arms flanking three BGCs are respectively selected as the adapter sequence (Figure 1). Searching the protospacer adjacent motifs (PAMs) in adapter sequence using CRISPR RGEN tools (http://www.rgenome.net/cas-designer).
   a. Select 5′-TTTV-3′ as the PAM for AsCpf1 from *Acidaminococcus* or LbCpf1 from *Lachnospiraceae*; 5′-TTN-3′ for FnCpf1 from *Francisella*; or the corresponding PAM for any other Cas12a proteins.[4] In this protocol, LbCas12a (TOLOBIO, Catalog# 32108-01) is used.
   b. For crRNA design, the length of spacer sequence is set to 17–19 bp.[4]
   c. Run the CRISPR RGEN tool and select spacers with high scores as candidates.

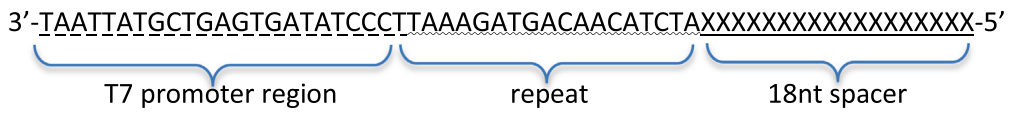
Note: The cleavage activity of Cas12a is affected by different spacer sequences.[15] To obtain the crRNA with better activity, this protocol suggests at least 3 spacer candidates at each

Table 1. Oligonucleotides used for *in vitro* transcription of crRNAs

Oligonucleotides	Sequence (5' → 3')	crRNA product
T7-oligo-F:	ATTAATACGACTCACTATAGGG	
49kb-spacer-oligo-US-R:	GTACGCGGGCAGCGTGAGATCTACAACAGTAGAAATTCCCTATAGTGAGTCGTATTAAT	crRNA-49kb-1
49kb-spacer-oligo-DS-R:	GGACAGAGATTTCGCCAAATCTACAACAGTAGAAATTCCCTATAGTGAGTCGTATTAAT	crRNA-49kb-2
87kb-spacer-oligo-US-R:	CCTGGCCGCGCCCGCCCGATCTACAACAGTAGAAATTCCCTATAGTGAGTCGTATTAAT	crRNA-87kb-1
87kb-spacer-oligo-DS-R:	CACAGTTTCGGTCCTCGGATCTACAACAGTAGAAATTCCCTATAGTGAGTCGTATTAAT	crRNA-87kb-2
145kb-spacer-oligo-US-R:	GATGCGCCGCTCCATCGAATCTACAACAGTAGAAATTCCCTATAGTGAGTCGTATTAAT	crRNA-145kb-1
145kb-spacer-oligo-DS-R:	GCGGATGTACGCATTGTTATCTACAACAGTAGAAATTCCCTATAGTGAGTCGTATTAAT	crRNA-145kb-2

homology arm is selected during crRNA design. Therefore, the length of adapter sequence would be arbitrarily adjusted if the suitable crRNA can't be found.

3. Design the spacer oligonucleotides (e.g., X-spacer-oligo-US-R, X-spacer-oligo-DS-R; "X" indicated target BGC, Table 1) that used for crRNA *in vitro* transcription as indicated as follow:



Preparation of crRNA for Cas12a digestion

Timing: 6 h

This step describes the *in vitro* transcription of three crRNA pairs (crRNA-49kb-1/2, crRNA-87kb-1/2, crRNA-145kb-1/2) which respectively guide upstream and downstream homology arms of three BGCs for Cas12a digestion (Figure 1). These crRNA pairs will be used in following step 10 and step 25 (see step-by-step method details). Commercially available kits are applied in this step.[22] The pipette tips, tubes, buffers and other regents used in this step must be RNase-free (Unless otherwise specified, all subsequent experiments involving RNA will use RNase-free materials).

4. Conduct PCR annealing of spacer oligonucleotides (Table 1) and T7 oligonucleotides (T7-oligo-F: 5'-GAAATTAATACGACTCACTATAGGG-3') to generate three pairs of double strand crRNA templates (Figure 2). Thermo Fisher® Taq DNA Polymerase PCR Buffer (Catalog# 18067017) is used in this step.
   a. Prepare 75 μL PCR annealing master mix (see materials and equipment) for double strand crRNA DNA templates preparation (without spacer oligonucleotides).

   Note: 75 μL PCR annealing master mix is enough for six PCR annealing reactions. The volume of PCR annealing master mix can be adjusted according the number of double strand crRNA DNA templates.

   b. Distribute 11 μL PCR annealing master mix into six PCR-tube, respectively.
   c. Add 9 μL of spacer oligonucleotides (pre-diluted to 10 μM) per tube. Mix by vortexing and spin down PCR-tubes.
   d. Place the tubes into thermocycler (Catalog# GE4852T) and run PCR annealing program: initial denaturation at 94°C for 5 min and then cool down from 94°C to 20°C with 1°C decrease per 30 s.
   e. Keep the resulting crRNA templates at 4°C until further processing.

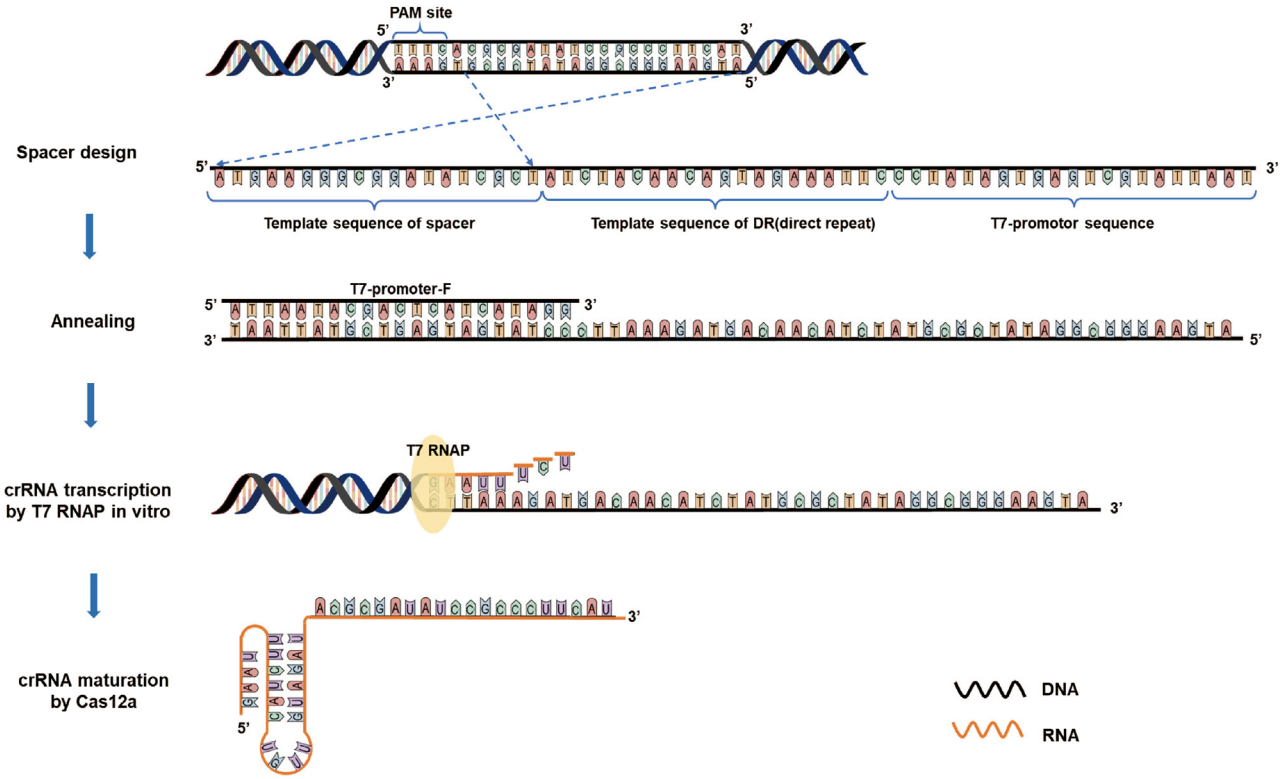


Figure 2. Schematic diagram and workflow for the preparation of crRNA by annealing and subsequent *in vitro* transcription

Spacer selection and spacer oligonucleotides design are performed according to step 2-3. The double strand crRNA DNA templates are generated by annealing of spacer oligonucleotides and T7 oligonucleotides (step 4). T7 RNA polymerase carries out the *in vitro* transcription in the presence of double strand crRNA DNA template, then the target crRNAs will be synthesized (step 5).

5. Perform *in vitro* transcription to generate crRNA pairs (Figure 2). NEB HiScribe™ T7 Quick High Yield RNA Synthesis Kit (NEB, Catalog# E2040S) is used in this step.
   a. Prepare 100 μL *in vitro* transcription master mix (see materials and equipment) for crRNA preparation.

   Note: 100 μL *in vitro* transcription master mix is enough for six *in vitro* transcription reactions. The volume of *in vitro* transcription master mix can be adjusted according the number of crRNA.

   b. Distribute 15 μL *in vitro* transcription master mix into six PCR-tubes, respectively.
   c. Add 5 μL of crRNA template (from step 4) per tube.
   d. Mix by vortexing and spin down the PCR tubes.
   e. Incubate the tubes at 37°C for 4 h.

   Optional: To increase the concentration of *in vitro* transcription product, the incubation time can be extended by an additional 1 or 2 h.

   f. After incubation, add 27 μL DNase/RNase-Free Distilled Water (ThermoFisher, Catalog# A57775) and 3 μL DNase I (6 units) (NEB, Catalog# M0303S) into each tube. Mix by vortexing and spin down the PCR tubes.
   g. Incubate the tubes at 37°C for another 15 min.
   h. Keep the resulting crRNA pairs at −20°C for up to one week, if necessary.

6. Remove the DNase and purify the resulting single strand crRNA (from step 5) by using RNA Clean & Concentrator TM-5 kit (Zymo Research, Catalog# R1013). The resulting three crRNA pairs (crRNA-49kb-1/2, crRNA-87kb-1/2, crRNA-145kb-1/2) will be used in following step 10 and step 25 (see step-by-step method details)

Note: The concentration of purified crRNA sample should be 1000–3000 ng/μL, the absorbance rations A260/280 should close to 2.0. If the concentration of the purified RNA is lower than 1000 ng/μL, the incubation time at step 5e could be extended accordingly.

Pause point: Keep the resulting purified crRNA at −20°C for one week. Long-term storage of the purified crRNA should keep at −80°C, if necessary.

Cell culture and mycelium preparation

Timing: 12–16 h

This step describes microorganism cell culture and preparation of mycelium for genomic DNA isolation. This mycelium sample will be used in following step 1 (see step-by-step method details). In this protocol, *S. albus* J1074 is cultured in Tryptone Soya Broth (TSB; Oxoid, Catalog# LA0020) medium.

7. Perform fresh spore suspension with 0.3 M sucrose solution in this step.
   a. Add 3 mL sterile 0.3 M sucrose solution to the plate.
   b. Scrape the surface of the culture with an inoculating loop to suspend the spores.
   c. Transfer the spore suspension into a sterile 1.5 mL microcentrifuge tube.
   d. Agitate the suspension on a vortex mixer for 1 min to break up spore chains.
8. 50 μL of freshly prepared spore suspension from step 7 is inoculated into a 250 mL shake flask containing 50 mL TSB medium (see materials and equipment) at 30°C, 220 rpm for 12 h.

Optional: Fresh mycelium also can be used for inoculation. TSB medium supplemented with 0.5% (w/v) glycine (Sigma, Catalog# V900144-500G) can be used for culture, if lysozyme could not treat the mycelium well at step 3 in 'Genomic DNA isolation by using agarose plugs'.

9. After 12 h cultivation, the mycelium at prophase of logarithmic phase is collected by centrifugation (Thermo Fisher Scientific, Catalog# 16-600-163) (4°C, 4000 × *g*, 5 min).

⚠ CRITICAL: At prophase of logarithmic phase, the mycelium with better dispersion, appeared to be smooth or loosely villiform without aggregation can be observed under a microscope is recommended by this protocol. The morphology of the mycelium is shown in upper of Figure 3. The rigid and dense mycelium clumps need to be avoided; fresh mycelium is recommended.

10. Add 1 mL 0.3 M sucrose buffer (see materials and equipment) to the tube contained the collected mycelium and pipet gently.
11. Centrifuge at 6, 000 × *g* for 3 min, discard the supernatant.
12. Repeat step 10 more two times.

Optional: The incubation time can be adjusted according the morphology of mycelium. The microorganisms with lower growth rate need an extended culture time accordingly.

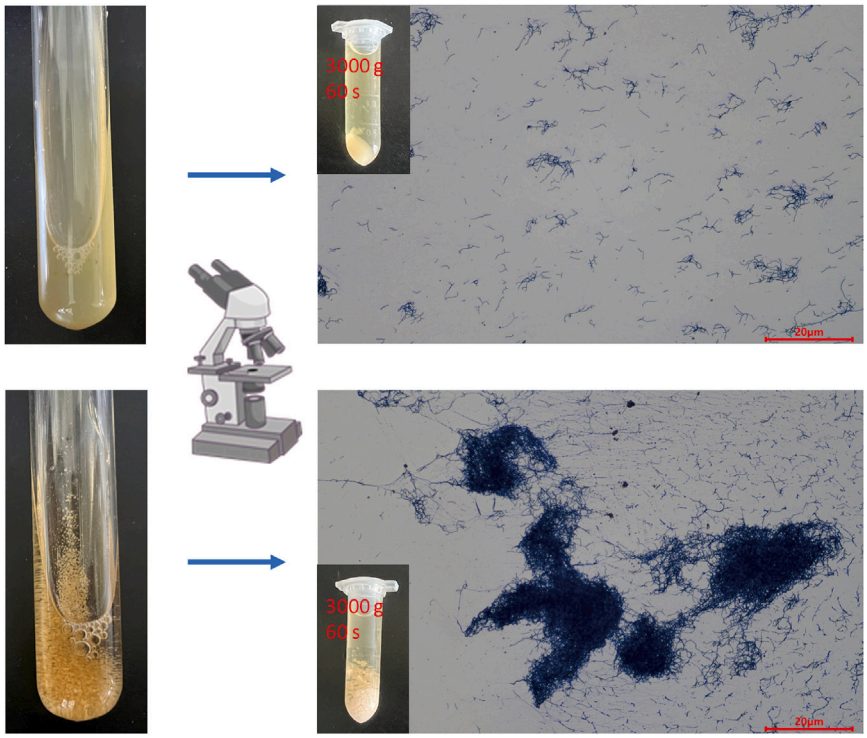


Figure 3. An illustration of different morphology of mycelium

The mycelium with better dispersion is shown in the upper, smooth or loosely villiform mycelium can be seen by a brief centrifuge (3000 × *g*, 60 s), and no mycelium aggregation can be found by microscopic examination. As shown in the bottom, the rigid and dense mycelium clumps can be seen by naked eyes, and the mycelium aggregation (> 20 μm) is clear under the microscope. Scale bar = 20 μm.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
Escherichia coli DH10B	Gibco BRL	Cat# 18297010
Escherichia coli EPI300	Tsingke	Cat# TSC-C10
S. albus J1074	Chater and Wilde[23]	DSM 41398
Chemicals, peptides, and recombinant proteins		
Chloramphenicol	Sangon Biotech	Cat# A600118;
X-gal	Yeasen	Cat# 10901ES03
IPTG	Yeasen	Cat# 10902ES10
Low melting point agarose	Sangon Biotech	Cat# A620014
Agarose	Sangon Biotech	Cat# A600015
Tryptone	Adamas	Cat# 89728B
Yeast extract	Adamas	Cat# 84106E
NaCl	Adamas	Cat# 81793CE
KCl	Adamas	Cat# 80636D
$MgCl_2$	Adamas	Cat# 82999H
D - (+)-Glucose	Adamas	Cat# 011137270
D - (+)-Sucrose	Adamas	Cat# 66841G
Dimethyl sulfoxide	Adamas	Cat# 75927R
1 M Tris-HCl buffer	Adamas	Cat# E8026-500 mL
L - (+)-Arabinose	Macklin	Cat# L824031

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Tris-Base	Solarbio	Cat# T8060
EDTA	Sigma	Cat# E6635-100G
$Na_2EDTA·2H_2O$	Macklin	Cat# E922480-100g
Glacial acetic acid	Greagent	Cat# G73562H
Boric acid	Aladdin	Cat# B111604-500g
SDS	Adamas	Cat# E8034-1kg
Potassium acetate	Greagent	Cat# G18899B
NaOH	Adamas	Cat# 19852G
Lysozyme	Sangon Biotech	Cat# A610308
Proteinase K	Biofrox	Cat# 1124MG100
PMSF	Beyotime	Cat# ST505
10 × NEBuffer™ 3.1	NEB	Cat# B7203
Deoxyribonuclease I (DNase I)	NEB	Cat# M0303S
TSB (Tryptone Soya Broth)	Oxoid Ltd	Cat# LA0020
Recombinant RNase inhibitor	Takara	Car# 2313A
Glycine	Sigma	Cat# V900144-500G
β-Agarase I	NEB	Cat# M0392s
LbCas12a	TOLOBIO	Cat# 32108-01
10,000× StarStain Red Nucleic Acid Dye	GenStar	Cat# E109
StarMarker 1Kb Ladder Plus	GenStar	Cat# M015-01
MidRange PFG Marker	NEB	Cat# N0342s
T4 DNA ligase	NEB	Cat# M0202s
DNase/RNase-free distilled water	Thermo Fisher	Cat# A57775
Critical commercial assays		
Taq DNA polymerase PCR buffer	Thermo Fisher	Cat# 18067017
KOD One™ PCR Master Mix	Toyobo	Cat# KMM-101S
ClonExpress II One Step Cloning Kit	Vazyme	Cat# C112-02
HiScribe™ T7 Quick High Yield RNA Synthesis Kit	NEB	Cat# E2050S
RNA Clean & Concentrator™-5 kit	Zymo Research	Cat# R1013
E.Z.N.A.® Plasmid Mini Kit I	Omega	Cat# D6943-02
2× *Taq* PCR StarMix with Loading Dye	GenStar	Cat# A012
Gel Cycle & pure kit	Omega	Cat# D2500
6× DNA loading buffer	GenStar	Cat# E106-10
Oligonucleotides		
crRNA preparation (see Table 1)	BGI-Shanghai	N/A
PCR primers for screening, verification, and modification (see Table 2)	BGI-Shanghai	N/A
Recombinant DNA		
pBAC2015	Wang et al.[19]	N/A
pUC19	NEB	Cat# N3041s
pBAC2015-49kb-J1074	N/A	N/A
pBAC2015-87kb-J1074	N/A	N/A
pBAC2015-145kb-J1074	N/A	N/A
Software and algorithms		
SnapGene		https://www.snapgene.com/
CRISPR RGEN tool		http://www.rgenome.net/cas-designer
Other		
NanoDrop 2000 spectrophotometer	Thermo Fisher	Cat# ND2000-EU
Thermal cycler	BIO-GENER	Cat# GE4852T
GelDoc XR + gel imaging system	Bio-Rad	Cat# 1708195
GenePulser Xcell™ system	Bio-Rad	Cat# 1652660
Microwave oven	Galanz	Cat# P70D20N1P-5(W0)
Microcentrifuge	Thermo Fisher	Cat# 16-600-163
Refrigerated centrifuge	SIGMA	Cat# Sigma 3–18K
Water bath	YIHENG	Cat# DHP-9082

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Shaker	ZHICHU Biotech	Cat# ZQWY-220V
CHEF-DR III Pulsed Field Electrophoresis Systems	Bio-Rad	Cat# 1703697
CHEF Disposable Plug Molds	Bio-Rad	Cat# 1703713
Clean Benches	SUZHOU SUJIE	Cat# SJ-CJ-1BQ
Electro-Thermal Incubator	YIHENG17	Cat# DHP9012
Eppendorf ThermoMixer® C	Eppendorf	N/A
50-well disposable plug mold	Bio-Rad	Cat# 1703713
CHEFDR III apparatus	Bio-Rad	Cat# 1703700
15 mL Falcon™ tube	Axygen	Cat# SCT-15ML-25-S

MATERIALS AND EQUIPMENT

- TSB medium

Dissolve 30 g Tryptone Soya Broth in ddH_2O to a 1000 mL total volume and sterilize it at 115°C for 20 min. Store at 20°C–25°C for up to one month.

- LB medium

Dissolve 10 g Tryptone, 5 g Yeast extract and 10 g NaCl in ddH_2O to a 1000 mL total volume and sterilize it at 115°C for 20 min. Store at 20°C–25°C for up to one month.

- SOC medium

Dissolve 20 g Tryptone, 5 g Yeast extract, 0.5 g NaCl, 0.186 g KCl, 0.952 g $MgCl_2$ and 3.6 g D - (+)-Glucose in ddH_2O to a 1000 mL total volume and sterilize it at 115°C for 20 min. Store at 20°C–25°C for up to one month.

- 10% Arabinose buffer

Dissolve 10 g L - (+)-Arabinose in ddH_2O to a 100 mL total volume and sterilize it using a 0.22 μM pore size syringe filter. Store at - 20°C for up to six months.

- 0.3 M sucrose

Dissolve 10.269 g D - (+)-Sucrose in ddH_2O to a 100 mL total volume and sterilize it at 115°C for 20 min. Store at 20°C–25°C for up to one month.

Table 2. Oligonucleotides used for candicidin BGC capture plasmid construction

Oligonucleotides	Sequence (5' → 3')
145kb-BAC-F	CCGGTATCCTCTCTATTTATTGGCTCCAAGTAGCGAAG
145kb-BAC-R	CCCGAGCATAGATAACGATGTGTCGGGTGCGGAGAAAG
145kb-US-F	CATCGTTATCTATGCTCGGGGGGCCGGGACGCTGAAG
145kb-US-R	CGGTTTGCGTATTGGGCAATCGGCTGCGCTCACGAGCCG
145kb-DS-F	GATAATAATGGTTTCTTAGGAAGGTGACGGCCACCGCCAG
145kb-DS-R	ATAAATAGAGAGGATACCGGGGCCTCGCTGCCGGGTCCGCTG
145kb-*LacZ*-F	ATTGCCCAATACGCAAAC
145kb-*LacZ*-R	CTAAGAAACCATTATTATC

PCR annealing master mix (one reaction)	
Reagent	Amount
10× PCR buffer	2 μL
T7-promoter-F (10 μM)	9 μL
Total	11 μL
Prepare before using.	

***In vitro* transcription master mix (one reaction)**	
Reagent	Amount
2× NTP buffer mix	10 μL
T7 RNA polymerase mix	2 μL
Recombinant RNase Inhibitor (40 U μL-1)	0.2 μL
DNase/RNase-Free Distilled Water	2.8 μL
Total	15 μL
Prepare before using.	

Cleavage master mix for DNA plug (one reaction)	
Reagent	Amount
10× NEBuffer 3.1	15 μL
LbCas12a (10 μM)	15 μL
Recombinant RNase Inhibitor (40 U μL-1)	1.5 μL
DNase/RNase-Free Distilled Water	118.5 μL
Total	150 μL
Prepare before using.	

PCR amplification master mix	
Reagent	Amount
2× KOD mix (1 U μL-1)	100 μL
Dimethyl Sulfoxide (5%)	10 μL
ddH_2O	90 μL
Total	200 μL
Prepare before using.	

PCR screening master mix (one reaction)	
Reagent	Amount
2× Taq PCR StarMix with Loading Dye	5 μL
ddH_2O	3.6 μL
Total	8.6 μL
Prepare before using.	

One Step Cloning master mix (one reaction)	
Reagent	Amount
5× buffer	4 μL
Exnase II	1 μL
ddH_2O	10 μL
Total	15 μL
Prepare before using.	

Digestion master mix for plasmid	
Reagent	Amount
10× NEBuffer 3.1	5 μL
LbCas12a (10 μM)	2 μL
Recombinant RNase Inhibitor (40 U μL-1)	0.5 μL
DNase/RNase-Free Distilled Water	30.5 μL
Total	38 μL
Prepare before using.	

Ligation mixture	
Reagent	Amount
T4 DNA ligase	2 μL
T4 DNA ligase buffer	4 μL
ddH_2O	10 μL
Total	16 μL
Prepare before using.	

TE25S buffer		
Reagent	Final concentration	Amount
1 M pH 8.0 Tris-HCl	25 mM	25 mL
0.5 M pH 8.0 EDTA	25 mM	50 mL
0.3 M Sucrose	0.3 mM	1 mL
H_2O	N/A	To 1000 mL
Total	**N/A**	**1000 mL**
Store at 20°C–25°C for up to six months.		

2.0% LMP agarose		
Reagent	Final concentration	Amount
Low melting point agarose	2%	2 g
10×TBE	0.5× TBE	5 mL
ddH_2O	N/A	To 100 mL
Total	**N/A**	**100 mL**
Store at 4°C for up to six months.		

Lysozyme solution		
Reagent	Final concentration	Amount
Lysozyme powder	2 mg/mL	50 mg
TE buffer	N/A	To 25 mL
Total	**N/A**	**25 mL**
Store at 4°C for up to one month.		

TE buffer		
Reagent	Final concentration	Amount
1 M pH 8.0 Tris-HCl	10 mM	5 mL
0.5 M pH 8.0 EDTA	1 mM	1 mL
ddH_2O	N/A	To 500 mL
Total	**N/A**	**500 mL**
Store at 20°C–25°C for up to six months.		

Proteinase K solution		
Reagent	Final concentration	Amount
Proteinase K powder	2 mg/mL	20 mg
NDS buffer	N/A	To 10 mL
Total	**N/A**	**10 mL**
Prepare before using.		

PMSF solution		
Reagent	Final concentration	Amount
Phenylmethanesulfonylfluoride	100 mM	174 mg
Isopropanol	N/A	To 10 mL
Total	**N/A**	**10 mL**
Store at 4°C for up to one month.		

⚠ CRITICAL: PMSF is highly toxic. Avoid direct contact with it during operation.

10× TBE buffer		
Reagent	Final concentration	Amount
Tris-Base	890 mM	54 g
$Na_2EDTA·2H_2O$	20 mM	3.72 g
Boric acid	890 mM	27.5 g
NaOH	Used for pH adjusting to 8.0	N/A
ddH_2O	N/A	To 500 mL
Total	**N/A**	**500 mL**
Store at 20°C–25°C for up to six months.		

50× TAE buffer		
Reagent	Final concentration	Amount
Tris	2 M	242 g
$Na_2EDTA·2H_2O$	100 mM	37.2 g
GLacial acetic acid	2 M	57.1 mL
ddH_2O	N/A	To 1000 mL
Total	**N/A**	**1000 mL**
Store at 20°C–25°C for up to six months.		

0.8% agarose		
Reagent	Final concentration	Amount
Agarose	0.8%	4 g
50× TAE	1× TAE	10 mL
StarStain Red Nucleic Acid Dye (10,000×)	N/A	10 μL
ddH_2O	N/A	To 500 mL
Total	**N/A**	**100 mL**
Prepare before using.		

0.8% LMP agarose		
Reagent	Final concentration	Amount
LMP Agarose	0.8%	0.8 g
10× TBE	0.5× TBE	5 mL

(*Continued on next page*)

Continued		
Reagent	Final concentration	Amount
StarStain Red Nucleic Acid Dye (10,000×)	N/A	10 μL
ddH_2O	N/A	To 100 mL
Total	**N/A**	**100 mL**
Prepare before using.		

Agarose gel for desalting		
Reagent	Final concentration	Amount
D - (+)-Glucose	2%	2 g
Agarose	1%	1 g
ddH_2O	N/A	To 100 mL
Total	**N/A**	**100 mL**
Store at 4°C for up to one month.		

Solution I		
Reagent	Final concentration	Amount
1 M Tris-HCl	25 mM	25 mL
0.5 M EDTA	10 mM	20 mL
20% D - (+)-Glucose	50 mM	45 mL
ddH_2O	N/A	To 1000 mL
Total	**N/A**	**1000 mL**
Store at 4°C for up to one month.		

⚠ CRITICAL: The solution needs to be sterilized by high temperature and high pressure at 121°C for 25 min. Before the experiment, 80 mg RNase I should be added into per 100 mL solution I.

Solution II		
Reagent	Final concentration	Amount
SDS	1%	1 g
2 M NaOH	200 mM	10 mL
ddH_2O	N/A	To 100 mL
Total	**N/A**	**100 mL**
Store at 20°C–25°C for up to six months.		

⚠ CRITICAL: Before using, 2 M NaOH and 10% SDS are mixed in the ratio of 1:1, which needs to be used immediately after mixing.

Solution III		
Reagent	Final concentration	Amount
Potassium acetate	3 M	147 g
GLacial acetic acid	5 M	57.5 mL
ddH_2O	N/A	To 500 mL
Total	**N/A**	**500 mL**
Store at 20°C–25°C for up to six months.		

NDS buffer		
Reagent	Final concentration	Amount
EDTA	0.5 M	14.61 g
Tris-HCl	10 mM	0.1211 g
SDS	1%	1 g
H_2O	N/A	To 100 mL
Total	N/A	100 mL
Store at 20°C–25°C for up to six months.		

STEP-BY-STEP METHOD DETAILS

Genomic DNA isolation by using agarose plugs

Timing: 3 h

This step describes the high-quality genomic DNA isolation by embedment of mycelium in low melting point agarose and subsequent in-gel DNA isolation.

1. Resuspend the prepared mycelium (from before you begin step 12) in TE25S (see materials and equipment) and then adjust the mycelium density with TE25S to the concentration of 100 mg/mL.
2. Mix 500 μL mycelium suspension with equal volume of 2.0% LMP agarose (see materials and equipment) and then pour into wells in a plug mold (100 μL). Place the mold at 4°C for 15 min.
3. Submerge the gel plugs into 10 mL prepared lysozyme solution (see materials and equipment), and incubate at 37°C for 30 min, and then discard the solution.
4. Transfer the plugs into 5 mL proteinase K solution (see materials and equipment) and incubate them at 50°C for 0.5–1 h, and then discard the solution.

Note: The agarose plugs should be transparent when the cells are completely lysed. The incubation time of proteinase K could be adjusted accordingly.

5. Transfer the plugs into 10 mL TE buffer adding 10 μL 100 mM PMSF solution (see materials and equipment) and incubate at 25°C for 30 min, and then discard the solution.
6. Rinse the plugs for 20 min with 10 mL TE buffer and place the tube on the decolorizing shaker (ZHICHU Biotech, Catalog# ZQWY-220V); Repeat this step three times.
7. Store the plugs in 70% ethanol at 4°C.

Pause point: The prepared genomic DNA agarose plugs can be stored in 70% ethanol at 4°C for up to 1 month.

Target BGC DNA fragment preparation

Timing: 3 h

This step describes the cut of the target BGC DNA fragment from the genomic DNA sample by using in-gel Cas12a digestion (Figure 4). The pipette tips, tubes, buffers and regents used in this step must be RNase-free.

8. Wash the plug (from step 7) with 1 mL DNase/RNase-Free Distilled Water for 10 min; Repeat this step three times
9. Equilibrate the plug in 1 mL 1 x NEB Buffer 3.1 at 25°C for 30 min.
10. Cut the target BGCs from genomic DNA sample by Cas12a under the guidance of crRNA pairs (from before you begin step 6).

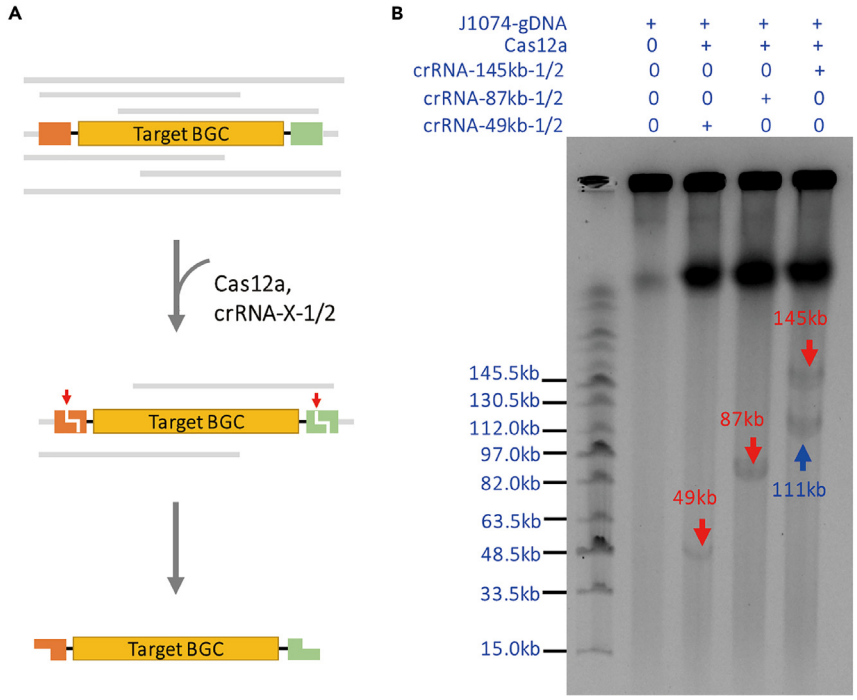

Figure 4. Cleavage of genomic DNA by Cas12a under the guidance of different crRNA pairs

(A) Cut the target BGC DNA fragment from the genomic DNA by Cas12a.

(B) Analyze the CRISPR/Cas12a-digested genomic DNA of *S. albus* J1074 by PFGE. Three pairs of crRNAs are used for genomic DNA digestion to obtain the resulting target DNA fragment. Bands of target BGC are indicated by red arrows. Band indicated by blue arrow is the end sequence of the genome, next to the 145kb gene cluster. The location showed in Figure 3. The figure was adapted from Liang et al. (2022).[1]

a. Submerge plug in 150 μL Cas12a cleavage master mix (see materials and equipment), then add 10 μL of X-US-crRNA (e.g., crRNA-145kb-1) and 10 μL of X-DS-crRNA (e.g., crRNA-145kb-2) (pre-diluted to 10 μM) and mix gently.

Note: 150 μL cleavage mix is only enough for one plug, the volume of mix can be adjusted according to the number of plugs.

b. Incubate the tube at 37°C for 1 h.

c. Discard liquid and rinse the plug in the tube with 1 mL DNase/RNase-Free Distilled Water (ThermoFisher, Catalog# A57775).

Optional: Cut 1/4 plug using a clean blade and run the pulsed field gel electrophoresis (PFGE) with GenePulser Xcell™ microbial system (Bio-Rad, Catalog# 1652662) to make sure that the target DNA fragment had been cleaved (Figure 4B). MidRange PFG Marker (NEB, Catalog# N0342s) is used as control. PFGE was performed in 0.5% agarose at 6 V/cm with a 1–25 s switching pulse time for 16 h in 0.5× TBE buffer at 14°C.

11. Hydrolysis the agarose plugs by β-Agarase I (NEB, Catalog# M0392s) and then release the BGC DNA fragment.
    a. Discard liquid in the tube and incubate at 70°C for 5 min to melt the agarose plug.
    b. Keep the melted agarose at 42°C for another 10 min.
    c. Add 5 μL β-Agarase I and mix very gently by very slowly invert the tube two to three times.

⚠ CRITICAL: After this step, the shearing of DNA fragment must be avoiding as much as possible, DO NOT mix by vortexing or pipetting.

d. Incubate the mixture at 42°C for 1 h.
e. Cool down the mixture at 4°C for 5 min, and examine the mixture, ensure the completely hydrolysis of the agarose gel.

Note: If the agarose gel can still be seen, please increase the amount of β-Agarase I and prolong the incubation time accordingly.

f. Store the sample at 4°C.

Pause point: the BGC fragment sample can be stored at 4°C for up to one day.

Purify target BGC DNA fragment by PFGE

Timing: 18 h

This optional step describes purification of resulting BGC DNA fragments from step 10 by PFGE.

12. Run PFGE with the agarose plug resulting from step 10.
    a. Melt the 0.8% LMP agarose gel (see materials and equipment) with Microwave oven (Galanz, Cat# P70D20N1P-5(W0)).
    b. Cast the melting 0.8% LMP agarose gel at 4°C for 15 min with a suitably sized comb.
    c. The agarose plug(s) from step 10 are inserted into the empty wells, and pressed firmly.
    d. The plugs are sealed by filling the wells with 0.8% LMP agarose.
    e. Run PFGE at 6 V/cm with a 1–25 s switching pulse time for 16 h in 0.5× TBE buffer at 14°C. MidRange PFG Marker is used.
13. Visualize the target DNA bands using a GelDoc XR + gel imaging system (Bio-Rad, Catalog# 1708195).
14. Recover the gel fraction containing target BGC DNA fragment with a clean blade.
15. Prepare the BGC DNA sample as described in step 11.

Pause point: the BGC fragment sample can be stored at 4°C for up to one day.

Capture plasmid construction and preparation

Timing: 36 h

This step describes the construction of the capture plasmid, and subsequent linearization of the capture plasmid by Cas12a digestion (Figure 5A). In this step, we take the preparation of the capture plasmid for the cloning of the 145-kb candicidin BGC as a demonstration.

16. Amplify BAC vector backbone, two homologous arms that flank target BGC, *LacZ* Cassette using KOD One TM PCR Master Mix (Toyobo, Catalog# KMM-101S).
    a. Prepare 200 μL PCR amplification master mix (see materials and equipment) for BAC vector backbone, homologous arms, and *LacZ* Cassette amplification.

Note: 200 μL PCR amplification master mix is enough for four PCR amplification reactions. And the volume can be adjusted according to the subsequent DNA usage.

    b. Distribute 44 μL PCR amplification master mix into 4 PCR-tubes, respectively.

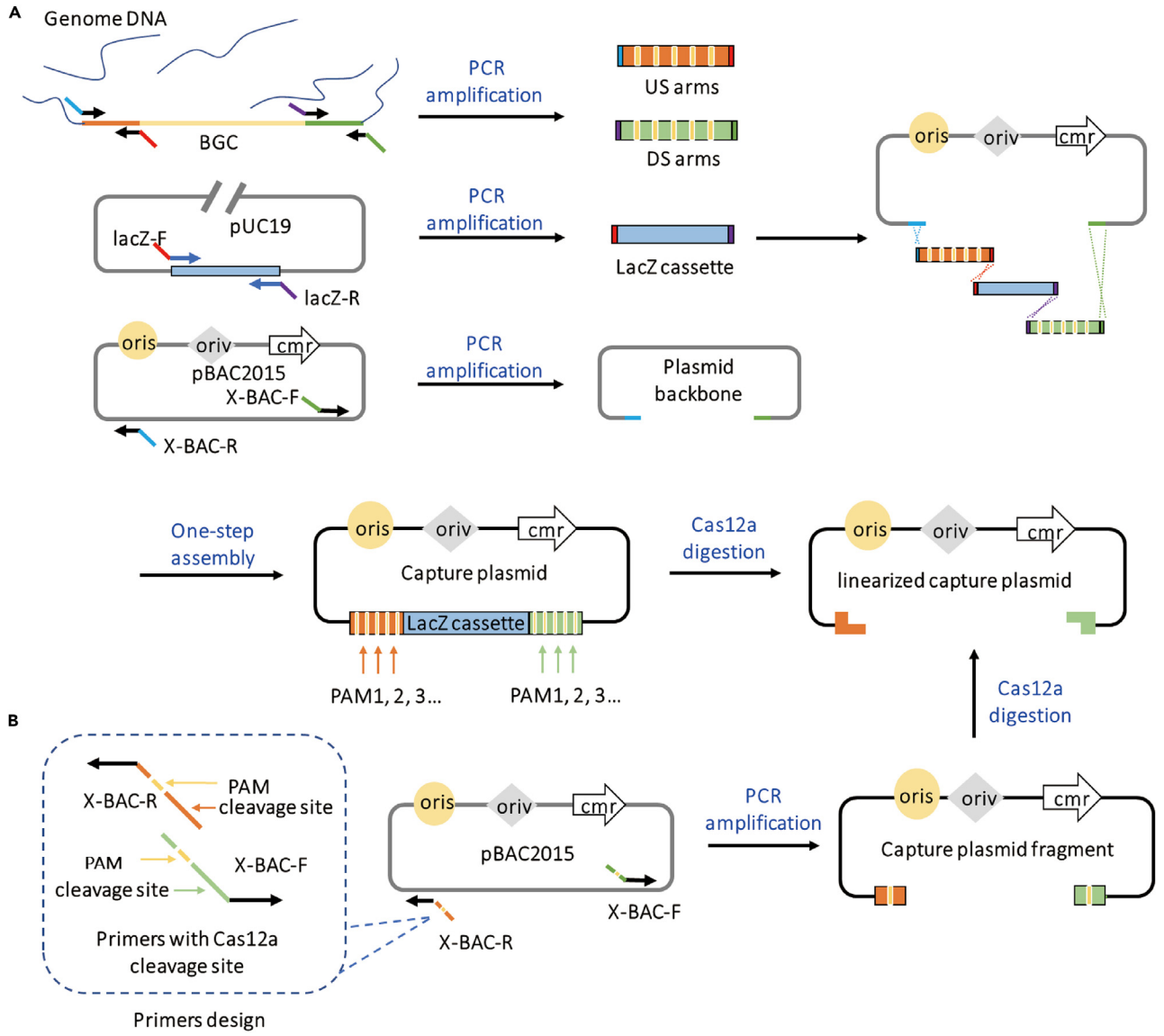

Figure 5. Capture plasmid construction and linearization

(A) Route A, the capture plasmid is constructed by one-step DNA assembly method, then the resulting capture plasmid is cleaved by Cas12a for linearization. A one-step recombination experiment was conducted using 100 ng plasmid backbone with ClonExpress II One Step Cloning Kit (Vazyme, Catalog# C112-02), contributing a conversion efficiency of approximately 100~200 CFUs and a positive rate of over 95%.

(B) Route B, the linearized capture plasmid is amplified by PCR and subsequently digested by Cas12a.

c. Add 2.5 μL of F-primer, 2.5 μL of R-primer (pre-diluted to 10 μM) and 1 μL corresponding template DNA (10 ng) into each PCR tube. Mix by vortexing and spin down PCR-tubes. The primers are listed below:

Note: pBAC2015 plasmid DNA, and 145kb-BAC-F/R primers are used for BAC vector backbone amplification; *S. albus* J1074 genomic DNA, and 145kb -US-F/R primers are used for upstream homology arm amplification; *S. albus* J1074 genomic DNA, and 145kb-DS-F/R primers are used for downstream homology arm amplification; pUC19 plasmid DNA, and 145kb-*LacZ*-F/R primers are used for *LacZ* Cassette amplification.

d. Place PCR tubes into a thermocycler and run PCR program as below:

PCR conditions

Step	Temperature (°C)	Time	Cycles
Initial denaturation	95	5 min	1
Denaturation	95	30 s	35 cycles
Annealing	60	30 s	
Extension	68	50 s*	
Final extension	72	5 min	1
Hold	20	5 min	1

Note: * The extension time could be adjusted according to the length of target PCR product.

e. Keep the resulting PCR products at 4°C until further processing.

17. Purify the resulting PCR products using a Nucleic acid purification kit (Omega, Catalog# D6942).
18. Measure the concentration of the resulting PCR products: BAC vector backbone, *LacZ* Cassette, upstream and downstream homologous arms by a NanoDrop 2000 spectrophotometer (Thermo Fisher, USA).

Note: The absorbance rations A260/280 should close to 1.8.

19. Construct target capture plasmid by homologous recombination by using ClonExpress II One Step Cloning Kit (Vazyme, Catalog# C112-02).
    a. Prepare 15 μL One Step Cloning master mix (see materials and equipment) for capture plasmid construction (without purified DNA fragment).
    b. Add 100 ng of BAC vector backbone, 12.5 ng of upstream homologous arm, 12.5 ng of downstream homologous arm and 12.5 ng of *LacZ* Cassette (pre-diluted to 30–50 ng/μL) per tube. Adjust the total volume to 20 μL.

Note: the volume of BAC vector, homologous arms, *LacZ* Cassette can be adjusting according the concentration of them under the guidance of the instruction.

    c. Incubate the tubes at 50°C for 10 min.
    d. Keep the resulting recombinant DNA at 4°C for short-term storage.

Pause point: The resulting recombinant DNA can be stored at -20°C for up to one week.

20. Transfer the recombinant DNA into *E. coli*.
    a. Pre-incubate the *E. coli* EPI300 chemical competent cells (Tsingke, Catalog# TSC-C10) on ice for 10 min.
    b. Transfer the resulting 20 μL recombinant DNA sample from step 19 into a sterilized EP tube.
    c. Add 50 μL EPI300 chemical competent cells into the tube. Mix gently and place the tube on ice for another 30 min.
    d. Heat shock the tube at 42°C for 45 s and quickly chill it on ice for 5 min.
    e. Add 1 mL fresh LB broth into the tube and recover the cells at 37°C, 220 rpm for 1 h.
    f. Centrifuge at 6, 000 × *g* for 1 min, discard the 900 μL of the supernatant and resuspend the cells in the rest LB broth.
    g. Spread 100 μL cell suspension onto a LB agar plate containing 30 μg/mL Chloramphenicol, 20 μg/mL X-Gal and 0.5 mM IPTG.
    h. Incubate the plate at 37°C for 12 h until colonies can be seen.

Note: The right colonies with the target capture plasmid are blue and resistant to Chloramphenicol, they can then be directly picked up from this LB agar plate.

21. Verify the selected right clones harboring the target capture plasmid by sequencing the upstream and downstream homology arms (plasmid sequencing service provided by BGI-Shanghai Ltd.).
22. Inoculate a validated colony into 5 mL LB broth supplemented with 30 μg/mL Chl and propagate the plasmid by growing the inoculum at 37°C, 220 rpm for 12 h. Add 50–100 μL 10% Arabinose buffer (see materials and equipment) to induce the duplication of the plasmid.
23. Isolate the resulting capture plasmid DNA from the culture from step 22 by using the E.Z.N.A.® Plasmid Mini Kit I (Omega, Catalog# D6943).
24. Measure the concentration of the capture plasmid DNA by NanoDrop 2000 spectrophotometer. And keep the resulting plasmid DNA at 4°C until further processing.

Note: The concentration of the isolated plasmid sample DNA should be adjusted to be 100–200 ng/μL using ddH_2O, and the absorbance rations A260/280 should close to 1.8.

Capture plasmid digestion and linearization

Timing: 2 h

This step describes the digestion of the capture plasmid DNA by Cas12a to generated complementary sticky ends with the target BGC DNA fragment (Figure 5A).

25. Cut the capture plasmid DNA by Cas12a under the guidance of crRNA pairs used in step 10.
    a. Prepare 38 μL digestion master mix (see materials and equipment) in EP tube for digestion of the capture plasmid (without crRNAs and capture plasmid).
    b. Add 1 μL of X-US-crRNA (e.g., crRNA-145kb-1) and 1 μL of X-DS-crRNA (e.g., crRNA-145kb-2) (pre-diluted to 10 μM) and 10 μL capture plasmid (pre-diluted to 100–200 ng μL^{-1}) per tube, and mix thoroughly.
    c. Incubate the tubes at 37°C for 30 min.
26. Add 10 μL 6× DNA loading buffer (GenStar, Catalog# E106-10) to 50 μL digested capture plasmid, mix thoroughly, and load the sample into a 0.8% agarose gel for conventional electrophoresis in 1× TAE buffer (see materials and equipment).
27. Recycle the gel fraction containing linear plasmid fragment by using a clean blade.
28. Purify and recycle the digested capture plasmid DNA by using Gel Cycle & Pure kit (Omega, Catalog# D2500).
29. Measure the concentration of the digested capture plasmid DNA by a NanoDrop 2000 spectrophotometer. Keep the resulting purified DNA at 4°C for a week.

Note: The concentration of plasmid sample should be adjusted to 50–100 ng/μL using ddH_2O, and the absorbance rations A260/280 must be close to 1.8.

Pause point: The resulting purified DNA can be stored at 4°C for one week.

Preparation of linear capture plasmid by PCR

Timing: 3 h

This optional step describes an alternative for linearization of the capture plasmid (Figure 5B). If the selected homology arm contains one PAM site, two 30-bp (4-nt PAM site, 26-nt target recognition and cleavage sequence) homology arms are also capable of BGC cloning.

30. Amplify linear capture plasmid fragment by using KOD One TM PCR Master Mix, as described in step 16.

Note: PCR template is pBAC2015 plasmid DNA, X-HA-BAC-F/R primers are used for BAC vector backbone amplification. As shown in Figure 5B. two 30-bp (4-nt PAM site, 26-nt target recognition and cleavage sequence) homology arms are incorporated into the primer sequence.

Note: Multiple primer pairs can be designed simultaneously to select the most efficient primer pair to meet the recognition specificity of Cas12a.

31. Add 3 μL DpnI (NEB, Catalog# R0176s) into each tube, incubate the system at 37°C for 60 min.
32. Purify the PCR product and measure the concentration as describe in step 17–18.
33. Linearize the capture plasmid by Cas12a to generated complementary sticky ends with the target BGC DNA fragment, as described in step 25–28.

Pause point: The resulting purified DNA can be stored at 4°C for one week.

BGC DNA fragment and linearized capture plasmid ligation, desalting and transformation

Timing: 14–16 h

This step describes the ligation, desalting and transformation of the resulting linearized capture plasmid and BGC DNA fragments by T4 DNA ligase.

34. Ligate BGC DNA fragments generated in step 11 (or step 15) and linearized capture plasmid generated in step 28 (or step 33) by T4 DNA ligase (NEB, Catalog# M0202s).
    a. Prepare 16 μL ligation mixture (see materials and equipment) for complete plasmid construction (without linearized capture plasmid and BGC DNA fragments).
    b. Add 20 μL of BGC DNA fragments generated in step 11 (or step 15) and 4 μL linearized capture plasmid generated in step 28 (or step 33) per tube with a wide-bore tip per tube.

Note: The shearing of DNA fragment must be avoided as much as possible, DO NOT mix by vortexing or pipetting.

    c. Incubate tubes at 16°C for 14–20 h.
    d. Keep the sample at 4°C for up to one day.
35. Preparation of desalter (Figure 6)
    a. Add 1.8 mL of desalt agarose gel (see materials and equipment) into a clean 2 mL microcentrifuge tube, place a 1.5 mL tube on the 2-mL tube to form a groove.
    b. Place the tubes at 4°C for 30 min.
    c. Remove the 1.5 mL tube and leave the solid groove.
36. Transfer 20 μL of the ligation product (Refer to step 34) into the groove of desalt agarose gel with a wide-bore tip.
37. Place the tube on ice for 1 h to remove the salt ions.
38. Electro-transformation of the ligation product into *E. coli* (Gibco, Catalog# 18297010) using the Gene Pulser Xcell Total System (Bio-Rad, Catalog# 1652660).

Note: The home-made electrocompetent *E. coli* cells can also be prepared according to a previous protocol by Dower et al.[24]

    a. Place a 50 μL electrocompetent *E. coli* cells (see materials and equipment) containing tube on ice for 30 min to thaw.

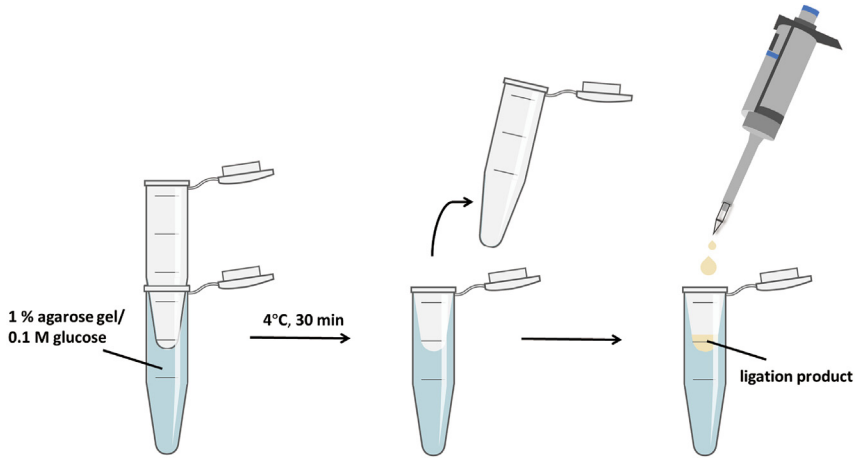

Figure 6. The schematic of preparation and use of desalter

b. Transfer 20 μL desalted ligation mixture from the groove into the electrocompetent cells containing tube with a wide-bore tip.
c. Transfer the mixture into a cold 0.2-cm cuvette (Bio-Rad, Catalog# 1652082EDU) on ice and tap the suspension to the bottom.
d. Electroporation is carried out with a GenePulser Xcell™ Mirobial system under the following conditions: C = 25 μF, PC = 200 Ω, V = 2.5 kV.
e. Take out the cuvette from the chamber and immediately add 1 mL fresh SOC medium (see materials and equipment).
f. Transfer the cell suspension into a sterilized 15 mL Falcon™ tube (Axygen, Catalog# SCT-15ML-25-S) and incubate at 37°C for 1 h with shaking at 220 rpm.
g. Spread the suspension on LB agar plates containing 30 μg/mL Chloramphenicol, 20 μg/mL X-Gal and 0.5 mM IPTG, and the colonies on plates can be obtained after incubate at 37°C for 12 h.

Positive clones PCR screening and verification

Timing: 15–18 h

This step describes screening of right clones that contains the target BGC, and subsequent verification by PFGE or restriction mapping.

39. Pick the white clones from LB plates (from step 38) and inoculate into EP tubes containing 500 μL LB medium with 30 μg/mL Chloramphenicol.
40. Incubate the tubes at 37°C for 4 h, 220 rpm.
41. Screen the right clones by a colony PCR protocol.
    a. Prepare the PCR screening master mix (see materials and equipment) (without DNA template).
    b. Distribute 9 μL PCR screening master mix into each PCR-tube.
    c. Add 1 μL of bacterial suspension of clone (from step 40) per tube, and mix thoroughly.
    d. Run PCR program with the conditions described in step 16d.
    e. Keep the resulting PCR product at 4°C until further processing.
42. The PCR product is analyzed by electrophoresis on 0.8% agarose gel and GelDoc XR + gel imaging system.
43. Inoculate 10 μL of each PCR-validated clones into an individual Falcon™ tube containing 10 mL LB liquid medium with 30 μg/mL Chloramphenicol, and incubate at 37 °C at 220 rpm for 12 h.

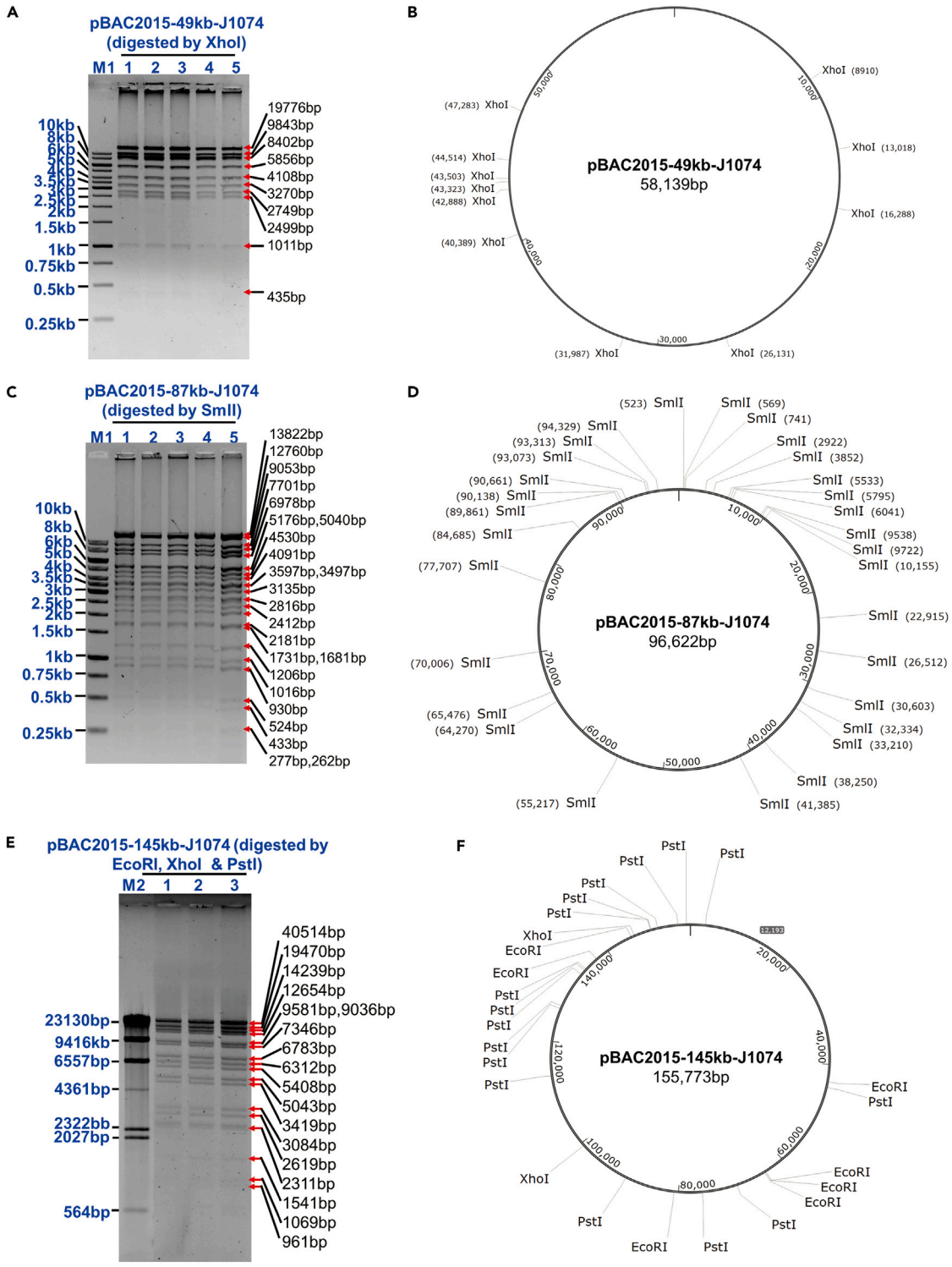

Figure 7. Validation of positive clones containing a paulomycin, surugamide or candicidin gene cluster

XhoI, SmlI and other restriction enzymes are used for paulomycin, surugamide and candicidin gene cluster restriction, respectively. Bands are indicated by arrows.

Figure 7. *Continued*

(A–F) A: pBAC2015–49 kb-J1074 digested by XhoI; B: schematic diagram of the position of the restriction sites of the plasmid pBAC2015–49 kb-J1074; C: pBAC2015–87 kb-J1074 digested by SmlI; D: schematic diagram of the position of the restriction sites of the plasmid pBAC2015–87 kb-J1074; E: pBAC2015–145 kb-J1074 digested by EcoRI, XhoI and PstI; F: schematic diagram of the position of the restriction sites of the plasmid pBAC2015–145 kb-J1074; M1, GeneRuler 1 kb DNA ladder (Thermo Fisher Scientific); M2, λ DNA-Hind III digestion marker (GenStar, China). The figure was adapted from Liang et al. (2022).[1]

44. Isolate plasmid contains the BGC of interest by an alkaline lysis protocol.
    a. Harvest cells by centrifugation 10 mL culture at 10, 000 × *g* for 1 min.
    b. Add 1 mL solution I contains RNase A (see materials and equipment), vortex or pipet up and down to mix thoroughly.
    c. Add 2 mL Solution II (see materials and equipment), invert and gently flip the tube several times to obtain a lysate.
    d. Add 1.5 mL Solution III (see materials and equipment), invert and gently flip the tube.
    e. Add 1.5 mL Chloroform (CAS:67-66-3), invert and gently flip the tube 8–10 times.
    f. Centrifuge the mixture at 10, 000 × *g* for 10 min.
    g. Transfer the upper aqueous solution into a new tube, add equal volume of isopropanol (CAS:67-63-0), mix gently by flipping.
    h. Centrifuge the mixture at 10, 000 × *g* for another 10 min.
    i. Discard the supernatant, add 1 mL 75% ethanol into the tube, mix it gently, then centrifuge the mixture at 10, 000 × *g* for 5 min.
    j. Repeat the step i two more times.
    k. Place the tube on the bench to dry naturally for 15 min (letting the ethanol to evaporate completely)
    l. Add 50 μL ddH_2O into the tube.
    m. Place the tube at 25°C for 10 min until the plasmid DNA is completely dissolved.
    n. Measure the concentration and purity using Use 1 μL of the plasmid by a NanoDrop 2000 spectrophotometer.

Pause point: The plasmid can be stored at −20°C for one month or 4°C for one week.

45. A secondary verification of the plasmid with the target BGC by restriction mapping (Figure 7).

EXPECTED OUTCOMES

By using this protocol, large DNA fragment of interest can be directly cloned from a given genomic DNA. As shown in Figure 7, three plasmid pBAC2015-49kb-J1074, pBAC2015-87kb-J1074 and pBAC2015-145kb-J1074, which respectively containing paulomycin, surugamide and candicidin BGC have been constructed by CAT-FISHING. Additionally, the captured BGCs can directly be used for heterologous expression in a suitable cell host, the potentially novel BSMs could be identified and characterized further.[1] Together with the well-established genome mining protocols, CAT-FISHING will surely facilitate the discovery of novel BSMs.

LIMITATIONS

In this protocol, a 145-kb DNA fragment with 75% GC content was cloned. To the best of our knowledge, this is the largest DNA fragment ever obtained by *in vitro* direct cloning from such high GC content genomic DNA samples. However, longer BGC DNA fragments, e.g., > 150 kb, 200 kb, or even larger have not yet been demonstrated by this method.

Previously, Enghiad et al. used CAPTURE (Cas12a-assisted precise targeted cloning using *in vivo* Cre-lox recombination) to clone BGCs ranging from 10 to 113 kb from both Actinomycetes and Bacill with ~100% efficiency.[20] Though the upper limit of cloning capacity is 113kb, the cloning efficiency of CAPTURE is the highest ever reported. Therefore, compared with CAPTURE, another limitation of our method is the efficiency. By using a simple and fast cloning route (without optional step 12-15),

the cloning efficiency of 145-kb DNA fragment with 75% GC content is ~10%. The cloning efficiency can raise to ~50%, if the optional steps is applied.

Lastly, this protocol combines the Cas12a-mediated precise cleavage and advanced features of bacterial artificial chromosome (BAC). Therefore, off-target of CRISPR/Cas12a and preparation and manipulation of genomic DNA agarose plugs also need to be concerned during the use of CAT-FISHING.

TROUBLESHOOTING

Problem 1

Low crRNA concentration after *in vitro* transcription (before you begin step 5–6).

Potential solution

Check the sequence of the T7 transcription template and RNase contamination; Extend the reaction time for transcription; Avoid RNase contamination during the whole experiment.

Problem 2

Mycelium clumping or aggregation (before you begin step 7–9).

Potential solution

Use fresh spore suspension for inoculation; Add glass beads or applying springs in shake flask to increase shear on the mycelium; Change medium or supplement with D - (+)-sucrose and glycine.

Problem 3

Agarose gel plug is whitish in color, and no transparent gel plugs after proteinase K treatment (step 4).

Potential solution

Use fresh bacterial cultures without mycelium clumping or aggregation; Prolong the reaction time of proteinase K.

Problem 4

No clear brands or smeared after PEGE (step 10).

Potential solution

The block and marker should be placed against the wall of the well and sealed with a low melting point agarose gel that melts at 25°C; Check the PFGE conditions; Ensure the high-quality genomic DNA sample in the plug.

Problem 5

No complete digestion of capture plasmid (step 25–27).

Potential solution

Prolong the reaction time; Adjust the ratio of Cas12a/crRNA and plasmid DNA; Change the crRNA pairs with higher activity.

Problem 6

No clone growth on selective LB agar plates (step 38).

Potential solution

Use pUC19 to test the activity of the electrocompetent cells, and the efficiency should be 10^9–10^{10} transformants per μg of plasmid DNA; Analysis the mixture resulting from step 10 by PFGE, make sure that the corresponding BGC DNA fragment is visible as shown in Figure 4B.

Problem 7

The clone growth but no positive clones (step 41–42).

Potential solution

See potential solutions to Problem 6; Pick and screening more white clones.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Gao-Yi Tan (tangy@ecust.edu.cn).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate any data or code.

ACKNOWLEDGMENTS

This work was financially supported by National Key Research and Development Program of China [2020YFA0907800, 2021YFC2100600, and 2020YFA0907304], the 111 Project (B18022), Fundamental Research Funds for the Central Universities [22221818014], and the Open Funding Project of the State Key Laboratory of Bioreactor Engineering.

AUTHOR CONTRIBUTIONS

G.Y.T. and L.Z. conceived and supervised the project. G.Y.T., F.X., and W.W. designed the experiments. M.L., X.Z., S.W., Y.J., L.L., and H.Y. performed the experiments and collected data. X.Z. and S.W. wrote the manuscript. Y.T. and G.Y.T. edited the manuscript.

DECLARATION OF INTERESTS

L.Z., G.Y.T., M.L., W.W., L.L., and X.Z. have filed one Chinese patent application (no. CN202010575747.5) based on this protocol.

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