Protocol

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This protocol presents an efficient genetic strategy to investigate gene function in the fungus *Aspergillus niger*. We combined 5S rRNA-CRISPR-Cas9 technology with Tet-on gene switch to generate conditional-expression mutants via precisely replacing native promoter with inducible promoter. We describe the design and DNA preparation for sgRNAs and donor DNA. We then detail the steps for DNA co-transformation into *A. niger* protoplasts by PEG-mediated transformation, followed by homozygote isolation. Finally, we describe the genome verification and strain validation of the isolates.

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

Protocol for gene characterization in Aspergillus niger using 5S rRNA-CRISPR-Cas9-mediated Tet-on inducible promoter exchange

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https://doi.org/10.1016/j.xpro.2022.101838

SUMMARY

This protocol presents an efficient genetic strategy to investigate gene function in the fungus Aspergillus niger. We combined 5S rRNA-CRISPR-Cas9 technology with Tet-on gene switch to generate conditional-expression mutants via precisely replacing native promoter with inducible promoter. We describe the design and DNA preparation for sgRNAs and donor DNA. We then detail the steps for DNA co-transformation into A. niger protoplasts by PEG-mediated transformation, followed by homozygote isolation. Finally, we describe the genome verification and strain validation of the isolates. For complete details on the use and execution of this protocol, please refer to Zheng et al. (2019).1

BEFORE YOU BEGIN

Filamentous fungi are of great economic importance as cell factories in the biotechnology industry, especially for bulk manufacturing of high-value products. 2–4 Owing to its high production capacity, secretion efficiency, and robust growth, Aspergillus niger has been widely exploited as workhorses for producing organic acids, proteins, and enzymes. 2,5 In spite of its industrial importance, due to its low frequency of homologous recombination, highly efficient genetic tools are limited, hampering the fundamental study of A. niger. 6 New genetic manipulation strategies that enable to characterize genes will contribute to key gene target discovery and validation for strain improvement of this vital fungal cell factory.

Herein, this protocol describes a detailed workflow to study the function of genes of interest by replacing its native promoter with the Tet-on inducible promoter 7,8 using the 5S rRNA-CRISPR/Cas9 technology.1 This strategy enables investigation of various phenotypes of conditional expression mutants caused by addition of the metabolite-independent inducer doxycycline (Dox). Briefly, we provide detailed instructions for sgRNAs and donor DNA design, co-transformation of sgRNA and donor DNA with a Cas9 encoding plasmid, to result in the desired Tet-on system exchange at the DNA double-strand break caused by Cas9 protein. Owing to the tight regulation of Tet-on switch by doxycycline, this technique is beneficial for gain-of-function and loss-of-function analysis.
using a single isolate, which obviated experimentally costly generation of multiple mutant strains in *A. niger*.

With this strategy, we have successfully generated several mutants of gene involved in the cell morphology, protein secretion, and citric acid production.\textsuperscript{9–13}

**sgRNACas9 software environment setting**

\textcircled{G} Timing: 0.5–1 h

1. Manually download and install Java (also known as Java Runtime Environment or JRE) from the website: http://www.java.com/en/.
2. Manually download and install Perl from the website: https://www.perl.org/get.html.
3. Manually download and install the latest version of the sgRNACas9 software\textsuperscript{14} (sgRNACas9_3.0.5) from website: http://www.biootools.com/software.html.

**Plasmid preparation**

\textcircled{G} Timing: 1–2 h

4. Inoculate *E. coli* Trans-T1 strains containing psgRNA6.0\textsuperscript{1} and pTC1.13\textsuperscript{10} plasmids in LB liquid media with 100 μg/mL Ampicillin and incubate at 37°C and 220 rpm for 16–20 h.
5. Extract the plasmid with a Miniprep kit (TIANGEN BIOTECH., Cat#DP103) according to the manufacturer’s handbook (TIANprep Mini Plasmid Kit_Plasmid DNA & DNA Clean Up_Product_TIANGEN).
6. Quantify plasmid concentration with the NanoDrop.
7. Store the plasmids at –20°C.

**Media and solution preparation**

\textcircled{G} Timing: 4–6 h

8. Prepare the LB agar plates\textsuperscript{15} and LB liquid media\textsuperscript{16} containing 100 μg/mL Ampicillin for *E. coli* using standard lab recipes (Cold Spring Harbor Protocols, 2009).
9. Prepare the CM liquid media, CM plates, MM plates and MMSN plates for *A. niger* using standard lab recipes.\textsuperscript{17} When necessary, add 150 μg/mL of hygromycin B for the selection marker hygromycin B phosphotransferase (\textit{hph}).

**KEY RESOURCES TABLE**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Bacterial and virus strains** |        |            |
| *E. coli* Trans-T1   | TransGen Biotech | Cat#CD501-02 |
| **Chemicals, peptides, and recombinant proteins** | | |
| Ampicillin           | Sigma-Aldrich     | Cat#A9393   |
| Hygromycin B         | Sigma-Aldrich     | Cat#V900372 |
| Doxycycline          | Sigma-Aldrich     | Cat#D3072   |
| Yeast extract powder | Formedium         | Cat#YEA02   |
| Agar                 | Formedium         | Cat#AGR10   |
| Tryptone             | Thermo Fisher Scientific | Cat#211705 |
| Casamino Acids       | Thermo Fisher Scientific | Cat#223050 |
| NaCl                 | Sigma-Aldrich     | Cat#S9888   |

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## Reagents or Resources

| Reagent or Resource | Source                     | Identifier   |
|---------------------|----------------------------|--------------|
| CaCl₂               | Sigma-Aldrich              | Cat#C4901    |
| MES                 | Sigma-Aldrich              | Cat#M3671    |
| Tris-HCl            | Sigma-Aldrich              | Cat#648313   |
| PEG-6000            | Sigma-Aldrich              | Cat#528877   |
| Sucrose             | Sigma-Aldrich              | Cat#V900116  |
| NaNO₃              | Sigma-Aldrich              | Cat#S5506    |
| KH₂PO₄             | Sigma-Aldrich              | Cat#P0662    |
| KCl                 | Sigma-Aldrich              | Cat#P3911    |
| MgSO₄·7H₂O          | Sigma-Aldrich              | Cat#230391   |
| ZnSO₄·7H₂O          | Sigma-Aldrich              | Cat#20251    |
| MnCl₂·4H₂O         | Sigma-Aldrich              | Cat#M3634    |
| CoCl₂·6H₂O         | Sigma-Aldrich              | Cat#C8661    |
| CuSO₄·5H₂O         | Sigma-Aldrich              | Cat#C8027    |
| Na₂MoO₄·2H₂O       | Sigma-Aldrich              | Cat#M1003    |
| Uridine            | Sigma-Aldrich              | Cat#U3003    |
| Sorbitol           | Sigma-Aldrich              | Cat#P8106    |
| Ethylenedinitrilotetraacetic acid (EDTA) | Sigma-Aldrich      | Cat#M6758    |
| Lysing Enzyme      | Sigma-Aldrich              | Cat#L1412    |

## Critical Commercial Assays

| Critical commercial assay | Source                          | Identifier   |
|--------------------------|---------------------------------|--------------|
| Tianprep Mini Plasmid Kit | TIANGEN BIOTECH                 | Cat#DP103    |
| Tianquick Mini Purification Kit | TIANGEN BIOTECH             | Cat#DP203    |
| Genomic DNA extract kit  | TIANGEN BIOTECH                 | Cat#DP305    |
| T4 DNA Ligase Reaction Buffer | New England Biolabs          | Cat#B0202S   |
| T4 DNA Ligase            | New England Biolabs             | Cat#M0318S   |
| Bsll                      | New England Biolabs             | Cat#R035S    |
| FastPfu Fly PCR SuperMix  | TransGen Biotech                | Cat#AS231-01 |

## Experimental Models: Organisms/strains

| Organism/strain                  | Source                                | Identifier   |
|----------------------------------|---------------------------------------|--------------|
| Aspergillus niger: Strain background: D353 | Shanghai Industrial Microbiology Institute Tech. Co. | SIMI: M203   |

## Oligonucleotides

| Oligonucleotide   | Source                          | Identifier   |
|-------------------|---------------------------------|--------------|
| sgRNA-pyrG-F      | Zhang et al.                    | caccGTAGTAGTTCCAGATTTTCGAC |
| sgRNA-pyrG-R      | Zhang et al.                    | aaccGTCCAGAACCTTGAACCTTCACCT |
| MH1-sgRNA-pyrG-F  | Zhang et al.                    | gtatccggccagctcttgataggtaagcatcag |
| MH1-sgRNA-pyrG-R  | Zhang et al.                    | ggtccgaacGCGTTAACCCTGATATTTGAAAG |
| pyrG-g-F          | Zhang et al.                    | ggacccgcagtgtagtgctactgacccgacttg |
| pyrG-g-R2         | Zhang et al.                    | CATGTCGACGGGAGATACAGAG |
| M13F               | Zhang et al.                    | GAGGCCCTAATGCCCTCAATGTGTC |
| M13R               | Zhang et al.                    | TTTAAAACGCAGCGCCAGT |

## Recombinant DNA

| Recombinant DNA | Source                          | Identifier   |
|-----------------|---------------------------------|--------------|
| psgRNA6.0       | Zheng et al.                    | N/A          |
| psgRNA6.15      | Zheng et al.                    | N/A          |
| pCas9-hph       | Zhang et al.                    | N/A          |
| pTC1.13         | Cairns et al.                   | N/A          |

## Software and Algorithms

| Software and algorithm | Source                          | Identifier   |
|------------------------|---------------------------------|--------------|
| sgRNA Cas9             | Xie et al.                      | http://www.biootools.com |
| FungiDB                | Stajich et al.                  | https://fungidb.org |
| Java                   | Java Software Foundation        | http://www.java.com |
| Perl                   | Perl Software Foundation        | https://www.perl.org |

## Other

| Equipment               | Source                          | Identifier   |
|------------------------|---------------------------------|--------------|
| NanoDrop 2000 Spectrophotometer | Thermo Scientific             | Cat#ND-2000  |
| Thermal Cycler         | Bio-Rad                         | Cat#186-1096 |
| Miracloth              | Calbiochem                      | Cat#4758SS   |

*All the recombinant plasmids are available on request.*
# MATERIALS AND EQUIPMENT

## LB liquid medium

| Reagent     | Final concentration | Amount |
|-------------|---------------------|--------|
| Yeast extract | 0.5% (w/v)         | 5 g    |
| Tryptone    | 1% (w/v)            | 10 g   |
| NaCl        | 1% (w/v)            | 10 g   |
| ddH₂O       | N/A                 | to 1 L |

Autoclave at 121°C for 20 min and add supplements afterward before use

100 mg/mL ampicillin 100 µg/mL 1 mL

Store at room temperature (RT, 25°C). Stable for one month.

## LB agar plate

| Reagent     | Final concentration | Amount |
|-------------|---------------------|--------|
| Yeast extract | 0.5% (w/v)         | 5 g    |
| Tryptone    | 1% (w/v)            | 10 g   |
| NaCl        | 1% (w/v)            | 10 g   |
| Agar        | 1.2%                | 12 g   |
| ddH₂O       | N/A                 | to 1 L |

Autoclave at 121°C for 20 min and add supplements afterward before use

100 mg/mL ampicillin 100 µg/mL 1 mL

Store at RT (25°C). Stable for one month.

## SMC buffer

| Reagent     | Final concentration | Amount |
|-------------|---------------------|--------|
| Sorbitol    | 1.33 M              | 242.32 g |
| CaCl₂ (5 M) | 50 mM               | 10 mL  |
| MES buffer  | (200 mM, pH 5.8)    | 100 mL |
| ddH₂O       | N/A                 | to 1 L |

Filter-sterilize. Store at 4°C. Stable for three months.

## TC buffer

| Reagent     | Final concentration | Amount |
|-------------|---------------------|--------|
| Sorbitol    | 1.33 M              | 242.32 g |
| CaCl₂ (5 M) | 50 mM               | 10 mL  |
| Tris-HCl buffer (1 M, pH 7.5) | 10 mM | 10 mL |
| ddH₂O       | N/A                 | to 1 L |

Filter-sterilize. Store at 4°C. Stable for three months.

## STC buffer

| Reagent     | Final concentration | Amount |
|-------------|---------------------|--------|
| Sorbitol    | 1.33 M              | 242.32 g |
| CaCl₂ (5 M) | 50 mM               | 10 mL  |
| Tris-HCl buffer (1 M, pH 7.5) | 10 mM | 10 mL |
| ddH₂O       | N/A                 | to 1 L |

Filter-sterilize. Store at 4°C. Stable for three months.
**Protoplast lysis buffer**

| Reagent      | Final concentration | Amount  |
|--------------|---------------------|---------|
| Lysing enzyme* | 0.2%                | 0.2 g   |
| SMC buffer   | N/A                 | to 10 mL|

Filter-sterilize. Freshly prepare.

*Alternatives:* Yatalase Enzyme of (Takara, Cat#T017) can be used as the alternative of Lysing enzyme of Sigma-Aldrich (Cat#L1412).

**PEG-6000 buffer**

| Reagent      | Final concentration | Amount  |
|--------------|---------------------|---------|
| PEG-6000     | 25%                 | 5.0 g   |
| TC buffer    | N/A                 | to 20 mL|

Filter-sterilize. Freshly prepare.

**50× ASP+N**

| Reagent      | Final concentration | Amount  |
|--------------|---------------------|---------|
| NaNO₃        | 3.5 M               | 60.00 g |
| KH₂PO₄       | 350 mM              | 14.97 g |
| KCl          | 350 mM              | 5.22 g  |
| ddH₂O        | N/A                 | to 200 mL|

Filter-sterilize. Store at RT (25°C). Stable for one month.

**100× Uridine**

| Reagent      | Final concentration | Amount  |
|--------------|---------------------|---------|
| Uridine      | 1 M                 | 24.42 g |
| ddH₂O        | N/A                 | to 100 mL|

Filter-sterilize. Store at 4°C. Stable for one month.

**1000× Trace Element**

| Reagent      | Final concentration | Amount  |
|--------------|---------------------|---------|
| EDTA         | 1.00%               | 10.00 g |
| ZnSO₄·7H₂O    | 0.44%               | 4.40 g  |
| MnCl₂·4H₂O    | 0.10%               | 1.01 g  |
| CoCl₂·6H₂O    | 0.032%              | 0.32 g  |
| CuSO₄·5H₂O    | 0.032%              | 0.32 g  |
| Na₂MoO₄·H₂O   | 0.03%               | 0.30 g  |
| CaCl₂        | 0.11%               | 1.11 g  |
| FeSO₄·7H₂O    | 0.10%               | 1.00 g  |
| ddH₂O        | N/A                 | to 1 L  |

Filter-sterilize. Store at RT (25°C). Stable for one month.

**CM liquid medium**

| Reagent      | Final concentration | Amount  |
|--------------|---------------------|---------|
| Casamino acids | 0.1%              | 1.0 g   |
| Yeast extract  | 0.5%              | 5.0 g   |
| ddH₂O        | N/A                 | to 1 L  |

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### Reagent Final concentration Amount

**Autoclave at 121°C for 20 min and add supplements afterward before use**

| Reagent                              | Final concentration | Amount |
|--------------------------------------|---------------------|--------|
| ASP+N (50×)                          | 1×                  | 20 mL  |
| Glucose (50%)                        | 1%                  | 20 mL  |
| MgSO₄·7H₂O (1 M)                     | 2 mM                | 2.0 mL |
| Trace elements (1000×)               | 1×                  | 1.0 mL |

Store at RT (25°C). Stable for one month.

### CM plate

| Reagent                              | Final concentration | Amount |
|--------------------------------------|---------------------|--------|
| Casamino acids                       | 0.1%                | 1.0 g  |
| Yeast extract                        | 0.5%                | 5.0 g  |
| Agar                                 | 1.2%                | 12 g   |
| ddH₂O                                | N/A                 | to 1 L |

Autoclave at 121°C for 20 min and add supplements afterward before use

| Reagent                              | Final concentration | Amount |
|--------------------------------------|---------------------|--------|
| ASP+N (50×)                          | 1×                  | 20 mL  |
| Glucose (50%)                        | 1%                  | 20 mL  |
| MgSO₄·7H₂O (1 M)                     | 2 mM                | 2.0 mL |
| Trace elements (1000×)               | 1×                  | 1.0 mL |

Store at RT (25°C). Stable for one month.

### MM plate

| Reagent                              | Final concentration | Amount |
|--------------------------------------|---------------------|--------|
| Agar                                 | 1.2%                | 12 g   |
| ddH₂O                                | N/A                 | to 1 L |

Autoclave at 121°C for 20 min and add supplements afterward before use

| Reagent                              | Final concentration | Amount |
|--------------------------------------|---------------------|--------|
| ASP+N (50×)                          | 1×                  | 20 mL  |
| Glucose (50%)                        | 1%                  | 20 mL  |
| MgSO₄·7H₂O (1 M)                     | 2 mM                | 2.0 mL |
| Trace elements (1000×)               | 1×                  | 1.0 mL |

Store at RT (25°C). Stable for one month.

### MMSN-bottom plate

| Reagent                              | Final concentration | Amount |
|--------------------------------------|---------------------|--------|
| Sucrose                              | 0.95 M              | 162.60 g|
| Agar                                 | 1.2%                | 6.00 g |
| ddH₂O                                | N/A                 | to 500 mL |

Autoclave at 115°C for 30 min and add supplements afterward before use

| Reagent                              | Final concentration | Amount |
|--------------------------------------|---------------------|--------|
| ASP+N (50×)                          | 1×                  | 10 mL  |
| MgSO₄·7H₂O (1 M)                     | 2 mM                | 1 mL   |
| Trace elements (1000×)               | 1×                  | 0.5 mL |

Store at RT (25°C). Stable for one month.
STEP-BY-STEP METHOD DETAILS

sgRNA and donor DNA design

**Timing:** 1–2 h

The protospacers of sgRNA determines the location, efficiency and specify of DNA cleavage by Cas9 for genome editing. Thus, we suggest designing a specific protospacer according to off-target evaluation of putative sgRNA sequences using the sgRNACas9 software. sgRNACas9 enables the fast design of sgRNA target sequences with minimized off-target effects by predicting the genome-wide Cas9 potential off-target cleavage sites (POT).

1. Prepare the input files for sgRNACas9.
   a. Download the gene sequence containing 1,000 bp upstream of start codon from FungiDB in FASTA format.
   b. Download the reference genome sequence from FungiDB in FASTA format, for instance, download the genome file of *A. niger* CBS513.88 from https://fungidb.org/fungidb/app/downloads/release-58/AnigerCBS513-88/fasta/data/.

   **CRITICAL:** Please note that the sgRNACas9 software does not allow any white space in the file name or file path.

2. Design the protospacer sequences using sgRNACas9 with the default parameters.
   a. Choose the gene sequence file as the input of Target sequence and choose the genome sequence file as the input of Genome sequences (Figure 1A).
   b. There is no need to change parameters other than mandatory options (Figure 1A).
      i. Select sgRNA length: 20 nt;
      ii. GC%: 40%–60%;
      iii. Select appropriate DNA strands: Both DNA strand;
      iv. Type of sgRNA: Single sgRNA;
      v. If select the sgRNA type as paired-gRNAs (optional), offset distance of gRNAs: min: -2 and max: 32;
      vi. Maximum number of mismatches: 5.
   c. Click on “Run”.
   d. Please find the output file of sgRNACas9_report.xls in the “Report” folder. An example of the output is shown in Figure 1C.

3. Select protospacer candidates according to the following parameters:
   a. To replace the native promoter with Tet-on system, the position of the protospacer is better to be located within a 100-bp window of the upstream of the ATG starting codon (Figure 1C).
   b. To ensure the genome editing efficiency and specificity, there are some recommended criteria:

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### MMSN-Top medium

| Reagent                      | Final concentration | Amount   |
|------------------------------|---------------------|----------|
| Sucrose                      | 0.95 M              | 162.60 g |
| Agar                         | 0.6%                | 3.00 g   |
| ddH₂O                        | N/A                 | to 500 mL|

Autoclave at 115°C for 30 min and add supplements afterward before use

ASP+N (50×)                    | 1x                   | 10 mL    |
MgSO₄₇H₂O (1 M)                 | 2 mM                 | 1 mL     |
Trace elements (1000×)          | 1x                   | 0.5 mL   |

Store at RT (25°C). Stable for one month.
i. No POT sites or Risk_evaluation: Best;
ii. No more than 4 continuous T/A nucleotides (4,6 nucleotide poly (T) tract acts as a termination signal for RNA pol III);
iii. Homopolymer sequences (more than 5 continuous A or C or G, more than 6 dinucleotide or trinucleotide repeats);
iv. No Type IIS Enzyme restriction sites for GoldenGate cloning.

CRITICAL: The preferred PAM of spCas9 from Streptococcus pyogenes is NGG, but protospacers with PAM of NAG can also be cleaved with lower efficiency. To choose a specific protospacer, the off-target evaluation with NAG PAM should be taken into consideration. The definition of OT and POT is shown in Figure 1B. OT is the off-targets with mismatches less than 5 and POT is the off-targets with perfect match to the 12-nt seed region of the sgRNA and mismatches less than 5.

4. Design oligos for sgRNA cloning, based on the selected protospacer sequence (20-nt, without PAM) as follows:
a. Forward oligo: 5’-cacc-20-nt target sequence-3’.
b. Reverse oligo: 5’-aaac- reverse complement 20-nt target sequence-3’.

Example: sgRNA sequence: 5’-GAGTAGTTCGAAGTTTCGACTGG-3’.

20-nt target sequence PAM.

Forward oligo: 5’-caccGAGTAGTTCGAAGTTTCGAC-3’.

Reverse oligo: 5’-aaacGTCGAAACTTCGAACTACTC-3’.

5. Design oligos for donor DNA PCR amplification, based on the selected protospacer sequence (20-nt, without PAM).
   a. Forward oligo is comprised of 40-nt upstream homologous arm and specific amplified primer sequence for the template. Upstream homologous arm could be located within the 100 upstream of the target sequence, to ensure the homologous recombination efficiency.
   b. Reverse oligo is comprised of 40-nt homologous arm started from ATG starting codon and the specific amplified primer sequence for the template.

sgRNA plasmid cloning

© Timing: 2–3 days

The sgRNA expressing plasmids are constructed by digestion of sgRNA expression plasmids psgRNA6.0 with BbsI, and ligation with annealed overlapping complementary oligos (Figure 1D).

6. Digest psgRNA6.0 with BbsI.
   a. Prepare the digestion mix in a PCR tube:

| Digestion reaction master mix | Amount |
|------------------------------|--------|
| 10× Digestion buffer         | 5 µL   |
| BbsI                        | 5 µL   |
| psgRNA6.0                  | 20 µL (5 µg) |
| ddH2O                      | 20 µL   |
| Total                      | 50 µL   |

b. Perform the digestion reaction in a thermocycler at 37°C for 2 h.
   c. Purify the digested psgRNA6.0 fragment with DNA purification kit (TIANGEN Biotech., Cat#DP203) following the manufacturer’s handbook (TIANquick Mini Purification Kit_Plastid DNA & DNA Clean Up_Product_TIANGEN).

7. In vitro oligos annealing:
   a. Prepare a 100 µM dilution of the oligos in ddH2O.
   b. Mix 10 µL forward oligos (100 µM) and 10 µL reverse oligos (100 µM) in a PCR tube.
   c. Anneal the oligos in a thermocycler using the following program:

| In vitro oligos annealing conditions | Temperature | Time |
|-------------------------------------|-------------|------|
| Denaturation                        | 95°C        | 5 min|
| Annealing                           | 25°C, -0.1°C/s | 10 min|
| Hold                                | 4°C         | forever|
8. Ligate the annealed overlapping complementary oligos with digested plasmid psgRNA6.0.
   a. Prepare a 1:100 dilution of the sgRNA overlapping oligos obtained in step 7.
   b. Prepare the ligation mix in a PCR tube:

   | Ligation reaction master mix | Amount |
   |-----------------------------|--------|
   | 10×T4 buffer                | 2 µL   |
   | T4 ligase                   | 2 µL   |
   | 100× diluted sgRNA fragment from step 7 | 1 µL |
   | psgRNA6.0                   | 2 µL (200 ng) |
   | ddH₂O                       | 13 µL  |
   | Total                       | 20 µL  |

c. Perform the ligation reaction in a thermocycler at 22°C for 4 h.

9. Transform 5 µL of ligation mix in 50 µL CaCl₂ competent cells and plate on the LB plate containing ampicillin.

△ CRITICAL: To exclude the influence of transformation efficiency, the plasmid psgRNA6.0 and its digested fragments were used as positive and negative transformation controls, simultaneously transformed in in 50 µL CaCl₂ competent cells.

10. Pick up colonies and check the targeting DNA sequences of sgRNA cassette via Sanger dideoxy DNA sequencing to identify positive colonies.

11. Extract sgRNA expressing plasmid from the overnight (16 h) culture of the positive colonies with a Miniprep kit (TIANGEN BIOTECH., Cat#DP103) according to the manufacturer’s handbook (TIANprep Mini Plasmid Kit_Plasmid DNA & DNA Clean Up_Product_TIANGEN).

**DNA preparation for sgRNA and donor DNA**

指向: 4–6 h

For DNA transformation into A. niger protoplast, the sgRNA expressing cassette and donor DNA fragment are obtained by PCR amplification.

12. Amplify the sgRNA expressing cassette and donor DNA fragment via PCR with either psgRNA or pTC1.13 as template and with M13F/M13R or MH-F/MH-R as primers, respectively.
   a. Prepare the PCR mix:

   | PCR reaction master mix | Amount |
   |-------------------------|--------|
   | 2×FastPfu Fly PCR SuperMix | 25 µL |
   | MH-F (10 µM)            | 2.5 µL |
   | MH-R (10 µM)            | 2.5 µL |
   | pTC1.13 (10 ng/µL)      | 1 µL   |
   | ddH₂O                   | 19 µL  |
   | Total                   | 50 µL  |

b. Perform the PCR reaction in the thermocycler with the following program:

| PCR cycling conditions | Temperature | Time | Cycles |
|------------------------|-------------|------|--------|
| Initial Denaturation   | 98°C        | 30 s | 1      |
| Denaturation           | 98°C        | 15 s | 30 cycles |

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13. Purify PCR products of sgRNA and donor DNA with DNA purification kit (TIANGEN BIOTECH., Cat#DP203) following the manufacturer’s handbook (TIANquick Mini Purification Kit_Plasmid DNA & DNA Clean Up_Product_TIANGEN).

14. Measure DNA concentration with the NanoDrop. The concentration of sgRNA and donor DNA is recommended to be more than 500 ng/μL.

15. Store the purified PCR products at –20°C and proceed with A. niger protoplast transformation.

**DNA co-transformation into A. niger protoplasts**

© Timing: 3–5 days

Three DNA components, including Cas9 expressing plasmid, sgRNA expressing cassette and donor DNA fragment, are co-transformed into A. niger protoplasts by the approach of PEG-mediated transformation based on the Meyer et al. procedure.

16. Inoculate 2.5 × 10^8 spores in a flask with 250 mL of complete medium (CM) and incubate for 16–18 h at 30°C and 50 rpm.

17. Harvest the mycelia by filtration through sterile Miracloth (Calbiochem) and wash 10 mL with SMC.

18. Prepare 0.5 g (wet weight) of mycelia to 10 mL the protoplast lysis buffer, and incubate for 2.0–3.0 h 37°C with gentle shaking at 75 rpm in a shaker horizontally.

19. Check protoplast release using the microscope after 1.5 h of incubation and then every 30 min. An example of protoplast releasing from the mycelial cells has been shown in Figure 2.

20. Add 10 mL STC to the protoplastation sample, when the protoplast concentration reaches up to about 10^6–7/mL.

21. Harvest the protoplasts through a sterile Miracloth filter and centrifuge at 3,000 g for 10 min at 4°C.

22. Decant the supernatant, resuspend the protoplasts in 1 mL of STC and centrifuge for 5 min at 3,000 g.

23. Keep protoplasts on ice and transform DNA into protoplast:

| DNA transformation mix |
|------------------------|
| Reagent                | Amount      |
| Protoplasts (10^7–8/mL) | 100 μL      |
| sgRNA fragment (1 μg/μL) | 3 μL        |
| donor DNA fragment (1 μg/μL) | 3 μL       |
| pCas9-hph (1 μg/μL)    | 3 μL        |
| PEG-6000 buffer        | 25 μL       |

△ CRITICAL: PEG-6000 buffer is not more than 25 μL for 100 μL protoplasts.

△ CRITICAL: Instead of DNA fragments, add 9 μL STC buffer for negative control.
24. Incubate at room temperature for 5 min.
25. Add 1 mL PEG-6000 buffer and 2 mL STC buffer to DNA transformation mix.
26. After adding 15 mL MMSN-Top medium, pour onto the selective MMSN-Bottom plates supple-
mented 150 µg/mL Hygromycin B and 10 µg/mL Dox. To avoid the degradation of Dox, please
keep the MMSN-Bottom plates under dark.
27. Incubate selective transformation plates at 30°C for 3–6 days until colonies are visible.

Homozygote isolation

© Timing: 3–5 days

Due to the presence of heterokaryons in filamentous fungi, it is necessary to purify them before ge-
notype verification step.

28. Dip the spores of the transformant using a cotton swab and wash the spores in 5 mL sterile water
to make the diluted spore suspensions.
29. Subculture primary transformants for monoclonal cultivation by plating diluted spore suspen-
sions on CM plates containing 150 µg/mL Hygromycin B and 10 µg/mL Dox.
30. Pick up the single colony (Figure 3) and cultivate on CM plates containing 150 µg/mL Hygrom-
ycin B and 10 µg/mL Dox.
31. Repeat the monoclonal cultivation step twice for obtaining the homokaryons of each transform-
ant, until there are no unedited spores during genomic PCR verification.

Genomic PCR verification

© Timing: 1–2 days

To determine the genotype of transformant isolates, genomic DNAs of the isolates are extracted
and verified by the method of diagnostic PCR and sequencing analysis.

32. Inoculate spores of the isolates in the flask containing 250 mL CM media supplemented with
10 µg/mL Dox and incubate for 16–18 h at 30°C and 220 rpm.
33. Harvest the mycelia by filtration through filter paper and grind using liquid nitrogen.
34. Extract genomic DNAs of the isolates using Genomic DNA extract kit (TIANGEN BIOTECH.,
Cat#DP305) according to the manufacturer’s handbook (Plant Genomic DNA Kit_Genomic
DNA_Product_TIANGEN).

Figure 2. Protoplasts releasing from the mycelia of A. niger
The protoplasts released from the mycelia after treated with lysing enzyme. After incubation for 1.5 h, some
protoplasts and small hypha fragments appeared. When increased the treatment time for 2.5 h, a large number of a
large number of protoplasts were obtained. Black bar represents 10 µm.
35. Genomic DNA of the homozygotes verified via diagnostic PCR and sequencing analysis with the corresponding primers (Figure 4).
   a. Prepare the PCR mix:

   | Reagent                              | Amount |
   |--------------------------------------|--------|
   | 2×FastPfu Fly PCR SuperMix           | 10 μL  |
   | pyrG-g-F (10 μM)                     | 1 μL   |
   | pyrG-g-R (10 μM)                     | 1 μL   |
   | Genomic DNA (10 ng/μL)              | 1 μL   |
   | ddH₂O                               | 7 μL   |
   | Total                               | 20 μL  |

   b. Perform the PCR reaction in the thermocycler with the following program:

   | Steps            | Temperature | Time | Cycles |
   |------------------|-------------|------|--------|
   | Initial Denaturation | 98°C        | 30 s | 1      |
   | Denaturation      | 98°C        | 15 s | 30 cycles |
   | Annealing         | 55°C        | 20 s |        |
   | Extension         | 72°C        | 3 min* |        |
   | Final extension   | 72°C        | 5 min | 1      |
   | Hold              | 4°C         | forever |        |

   **Note:** Extension time is dependent on the expected size of PCR product and the extension efficiency of DNA polymerase. For example, the expected size of PCR product is about 5.5 kb and the amplification efficiency of FastPfu Fly DNA polymerase is 2–4 kb/min, so the extension time is set up as 3 min.

36. Load 5 μL PCR product on 1% agarose gel and verify PCR via DNA electrophoresis (Figure 4).
37. Select one or two verified transformant isolates and proceed with cell growth analysis.
Strain validation

Timing: 2–3 days

Due to various phenotypic change caused by different genes, here we take of pyrG conditional expressing mutant as an example to display the gene expression regulation by addition of the inducer Dox. For more details, please refer the protocol of quantitative phenotypic screens of A. niger mutants (Cairns et al., under revision).
38. Inoculate spores of pyrG conditional expressing isolates, with concentration of 500 spores/μL, 50 spores /μL, and 5 spores /μL in 2 μL volumes onto the MM plates supplemented with various concentrations of Dox (0, 0.2, 2, 20, 50 μg/mL) and MM plates with 10 mM uridine as positive control, respectively.

39. Incubate for 48 h at 30°C. To avoid the degradation of Dox, please keep the MM plates in a dark incubator.

40. Observe the growth of colonies under different concentrations of Dox (Figure 5). An example of pyrG titratable expression mutant was shown in Figure 4. The titration of Dox in MM agar without uridine enabled isolate XMD1.6 to prototrophy, and ultimately to generate colonies which resembled the parental strain, which indicated that the pyrG gene is essential to cell growth on MM plates without uridine.

EXPECTED OUTCOMES

CRISPR/Cas9 technology enables precise genome editing, provided that a suitable sgRNA and the proper donor DNA as repair template are employed. As mentioned above, conditional expressed mutants for gene function analysis are easily generated when targeted the upstream of start codon with the donor DNA containing Tet-on cassette (Figure 2). Here, this protocol consists of three parts: 1) sgRNA and donor DNA design; 2) DNA construct and co-transformation into A. niger protoplasts; 3) homozygote purification, genome verification and cell growth validation.

An example of sgRNA design is shown in Figure 1C. To ensure the efficiency and specificity of sgRNA, please follow the recommended suggestion in step 2. Considering the location of upstream of the start codon, the selected sgRNA is labeled in green front (Figure 1C), whose “Risk_evaluation” is “Best” and “Total_OT” is 3. The less “Total_OT”, the less risk of off-target. The appropriate sgRNA is the pre-requisite for precise gene editing.

Figure 2 gives an example of Protoplasts releasing from the mycelia of A. niger.

Figure 3 shows an example of the single colonies of subculture primary transformants of A. niger.

Figure 4 displays an example of successful Tet-on system replacement targeting the pyrG gene. The donor DNA containing the Tet-on cassette, were co-transformed with linear sgRNA construct and Cas9 expression plasmid into the protoplasts of A. niger D353. First, the sgRNA guides Cas9 protein
targeting the upstream of *pyrG* encoding sequence and cleavages the DNA double strand, generating double-strand break (DSB). An unrepaired DSB in *A. niger* is lethal. In the absence of the donor template, DNA repair occurs by the dominate error-prone non-homologous end joining (NHEJ) pathway. With the donor DNA as repair template, the DSB is repaired via homologous recombination with the integration of donor DNA, resulting in the replacement of native promoter by Tet-on inducible promoter (Figure 2). Thus, with the verified primers across the integration site, the correctly edited transformants can be very clearly identified after PCR amplification and electrophoresis of amplified products (Figure 2B).

*Figure 5* gives an example of validation of *pyrG* conditional expression mutants on solid plates. PyrG is essential for uridine synthesis. Thus, no growth of this conditional expression mutant was observed in the absence of Dox in growth media, and the mutant was indistinguishable from the control on the MM plate with uridine but without Dox. The addition of a gradient Dox in MM agar without uridine enabled isolate XMD1.6 to prototrophy, and ultimately to generate colonies which resembled the parental strain.

**LIMITATIONS**

The main limitation of CRISPR/Cas9 genome editing technology is the PAM preference. The necessity of PAM recognition constrains CRISPR-Cas9 systems for use across genomic loci that lack or only sparsely encode PAMs. Thus, some Cas9 variants xCas9,20 SpCas9-NG,21 SpG and SpRY,22 could circumvent this limitation by extending targeting to many sites with PAMs, Cas12a and other variants also might be worth to consider.

Due to the universality of CRISPR/Cas9 technology and Tet-on system, this protocol has can be applied for not only for *A. niger*, but for the other industrially, clinical or biotechnological relevant filamentous fungi. SgRNA expression is key limitation to the establishment of CRISPR/Cas9 technology in filamentous fungi. We have proven that the 5S rRNA promoter enables to high genome efficiency, when using for the sgRNA transcription.1 Therefore, the high-expression, wide distribution and high conservation make it easy to obtain the species specific 5S rRNA sequence.

**TROUBLESHOOTING**

**Problem 1**

No positive *E.coli* transformants containing the expected sgRNA (step 9).

**Potential solution**

- Double-check the oligos of sgRNA, whether the oligos are well complementary paired (step 4).
- Double-check the digested sgRNA expressing plasmid psgRNA6.0 (step 6). Check the DNA quality of the digested psgRNA6.0 via DNA electrophoresis on 1% agarose gel. Check the digestion of psgRNA6.0 from the negative transformation control (step 9).
- Dephosphorylate the digested psgRNA6.0 fragments by dephosphorylase to reduce the background interference.
- Please check the transformation efficiency of competent cells (step 9). The positive control using the plasmids psgRNA6.0 is used to confirm the transformation efficiency of competent cells.

**Problem 2**

Low concentration of protoplasts released from the mycelia (steps 16–19).

**Potential solution**

- Due to the difference in growth rate of *A. niger* strains, adjust the cultivation period of different strains (step 16), to ensure to lyase the young mycelia.
- Prepare the fresh protoplast lysing buffer.
Confirm the pH value of the lysing buffer as 5.8, to ensure the optimal pH of lysing enzyme (step 18).

Confirm the protoplastation temperature, to ensure the optimal temperature of lysing enzyme (step 18).

Problem 3
Low protoplast transformation efficiency (steps 23–27).

Potential solution
- Double-check the DNA fragments before protoplast transformation (steps 13 and 14).
- Double-check the DNA transformation system (step 23).
- Increase the amount of sgRNA, donor DNA and Cas9 plasmid (5–10 µg).

Problem 4
Unexpected genome editing in the transformants when verify the genotype (steps 34–37). The unexpected outcome might be caused by multiple DNA double-strand breaks pathways including NHEJ, microhomology-mediated end joining (MMEJ) or single-strand annealing (SSA).

Potential solution
- Double-check the PCR mix and add negative control.
- Double-check homozygote isolation.
- If necessary, please repeat the homozygote isolation step (steps 28–30).
- If possible, please use the NHEJ-deficient isolates, such as MA70.157 as hosts, to reduce the error-prone editing caused by the NHEJ pathway.
- To make the transformation easier and improve the transformation efficiency, sgRNA cassette and donor DNA could be subcloned into Cas9 expressing plasmid.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Ping Zheng (zheng_p@tib.cas.cn).

Materials availability
All unique/stable materials generated in this study will be made available upon request from the lead contact, but require a complete Materials Transfer Agreement.

Data and code availability
This study did not generate dataset or original code. Any additional information required is available from the lead contact upon request.

ACKNOWLEDGMENTS
This study was supported by Natural Sciences Foundation of China (32070082 and 31961133021), National Key R&D Program of China (2018YFA0900500), Tianjin Synthetic Biotechnology Innovation Capacity Improvement Project (TSBICIP-PTJS-003 and TSBICIP-UIJP-003), and by the Deutsche Forschungsgemeinschaft (DFG) for (Grant ME 2041/13-1).

AUTHOR CONTRIBUTIONS
Conceptualization, Methodology, Resources, and Writing – Original Draft, X.Z.; Validation, Visualization, X.Z.; Writing – Review & Editing, X.Z., T.C., P.Z., and V.M.; Funding Acquisition, X.Z. and J.S.; Supervision and Project Administration, P.Z. and J.S. All authors have approved the manuscript.
DECLARATION OF INTERESTS
The authors declare no competing interests.

REFERENCES

1. Zheng, X., Zheng, P., Zhang, K., Cairns, T.C., Meyer, V., Sun, J., and Ma, Y. (2019). SS rRNA promoter for guide RNA expression enabled highly efficient CRISPR/Cas9 genome editing in Aspergillus niger. ACS Synth. Biol. 8, 1568–1574.

2. Meyer, V., Fiedler, M., Nitsche, B., and King, R. (2015). The cell factory Aspergillus enters the big data era: opportunities and challenges for optimising product formation. Adv. Biochem. Eng. Biotechnol. 149, 91–132.

3. Boecker, S., Gratz, S., Kerwat, D., Adam, L., Schirmer, D., Richter, L., Schütze, T., Petras, D., Sussmuth, R.D., and Meyer, V. (2018). Aspergillus niger is a superior expression host for the production of bioactive fungal cyclodepsipeptides. Fungal Biol. Biotechnol. 5, 4.

4. Cairns, T.C., Ni, C., and Meyer, V. (2018). How a fungus shapes biotechnology: 100 years of Aspergillus Niger research. Fungal Biol. Biotechnol. 5, 13.

5. Tong, Z., Zheng, X., Tong, Y., Shi, Y.C., and Sun, J. (2019). Systems metabolic engineering for citric acid production by Aspergillus niger in the post-genomic era. Microb. Cell Fact. 18, 28.

6. Zheng, X., Zheng, P., Sun, J., Kun, Z., and Ma, Y. (2018). Heterologous and endogenous U6 snRNA promoters enable CRISPR/Cas9 mediated genome editing in Aspergillus niger. Fungal Biol. Biotechnol. 5, 2.

7. Meyer, V., Arentshorst, M., El-Ghezal, A., Drews, A.C., Koosra, R., van den Hondel, C.A.M.J.J., and Ram, A.F. (2007). Highly efficient gene targeting in the Aspergillus niger kusA mutant. J. Biotechnol. 128, 770–775.

8. Wanka, F., Cairns, T., Boecker, S., Berens, C., Happel, A., Zheng, X., Sun, J., Krappmann, S., and Meyer, V. (2016). Tet-on, or Tet-off, that is the question: advanced conditional gene expression in Aspergillus. Fungal Genet. Biol. 89, 72–83.

9. Cairns, T.C., Feurstein, C., Zheng, X., Zhang, L.H., Zheng, P., Sun, J., and Meyer, V. (2019). Functional exploration of co-expression networks identifies a nexus for modulating protein and citric acid titres in Aspergillus niger submerged culture. Fungal Biol. Biotechnol. 6, 18.

10. Cairns, T.C., Feurstein, C., Zheng, X., Zheng, P., Sun, J., and Meyer, V. (2019). A quantitative image analysis pipeline for the characterization of filamentous fungal morphologies as a tool to uncover targets for morphology engineering: a case study using aspID in Aspergillus niger. Biotechnol. Biofuels 12, 149.

11. Zhang, L., Zheng, X., Cairns, T.C., Zhang, Z., Wang, D., Zheng, P., and Sun, J. (2020). Disruption or reduced expression of the orotidine-5'-decarboxylase gene pyrG increases citric acid production: a new discovery during recyclable genome editing in Aspergillus niger. Microb. Cell Fact. 19, 76.

12. Cairns, T.C., Zheng, X., Feurstein, C., Zheng, P., Sun, J., and Meyer, V. (2021). A library of Aspergillus niger chassis strains for morphology engineering connects strain fitness and filamentous growth with submerged macromorphology. Front. Bioeng. Biotechnol. 9, 820088.

13. Zhang, X., Cairns, T.C., Ni, X., Zhang, L., Zhai, H., Meyer, V., Zheng, P., and Sun, J. (2022). Comprehensively dissecting the hub regulation of PkaC on high-productivity and pellet macromorphology in citric acid producing Aspergillus niger. Microb. Cell Fact. 21, 1867–1882.

14. Xie, S., Shen, B., Zhang, C., Huang, X., and Zhang, Y. (2014). sgRNAcas9: a software package for designing CRISPR sgRNA and evaluating potential off-target cleavage sites. PLoS One 9, e100448.

15. Cold Spring Harbor Protocols (2009). LB agar. Cold Spring Harb. Protoc. 3. pdb.rec11683.

16. Cold Spring Harbor Protocols (2016). LB liquid medium. Cold Spring Harb. Protoc. 9. pdb.rec09928.

17. Carvalho, N.D.S.P., Arentshorst, M., Jin Kwon, M., Meyer, V., and Ram, A.F.J. (2010). Expanding the ku70 toolbox for filamentous fungi: establishment of complementation vectors and recipient strains for advanced gene analyses. Appl. Microbiol. Biotechnol. 87, 1463–1473.

18. Sta¨jich, J.E., Harris, T., Brunk, B.P., Brestelli, J., Fischer, S., Harb, O.S., Kissinger, J.C., Li, W., Nayak, V., Pinney, D.F., et al. (2012). FungiDB: an integrated functional genomics database for fungi. Nucleic Acids Res. 40, D675–D681.

19. Hsu, P.D., Scott, D.A., Weinstein, J.A., Ran, F.A., Konermann, S., Agarwala, V., Li, Y., Fine, E.J., Wu, X., Shalem, O., et al. (2013). DNA targeting specificity of RNA-guided Cas9 nucleases. Nat. Biotechnol. 31, 827–832.

20. Hu, J.H., Miller, S.M., Geurts, M.H., Tang, W., Chen, L., Sun, N., Zeina, C.M., Gao, X., Rees, H.A., Lin, Z., and Liu, D.R. (2018). Evolved Cas9 variants with broad PAM compatibility and high DNA specificity. Nature 556, 57–65.

21. Nishimasu, H., Shi, X., Ishiguro, S., Gao, L., Hirano, S., Okazaki, S., Noda, T., Abudayyeh, O.O., Gootenberg, J.S., Mori, H., et al. (2018). Engineered CRISPR-Cas9 nucleases with expanded targeting space. Science 361, 1259–1262.

22. Walton, R.T., Christie, K.A., Whittaker, M.N., and Kleinsteve, B.P. (2020). Unconstrained genome targeting with near-PAMless engineered CRISPR-Cas9 variants. Science 368, 290–296.

23. Huang, J., and Cook, D.E. (2022). The contribution of DNA repair pathways to genome editing and evolution in filamentous pathogens. FEMS Microbiol. Rev. 18. 245–256.