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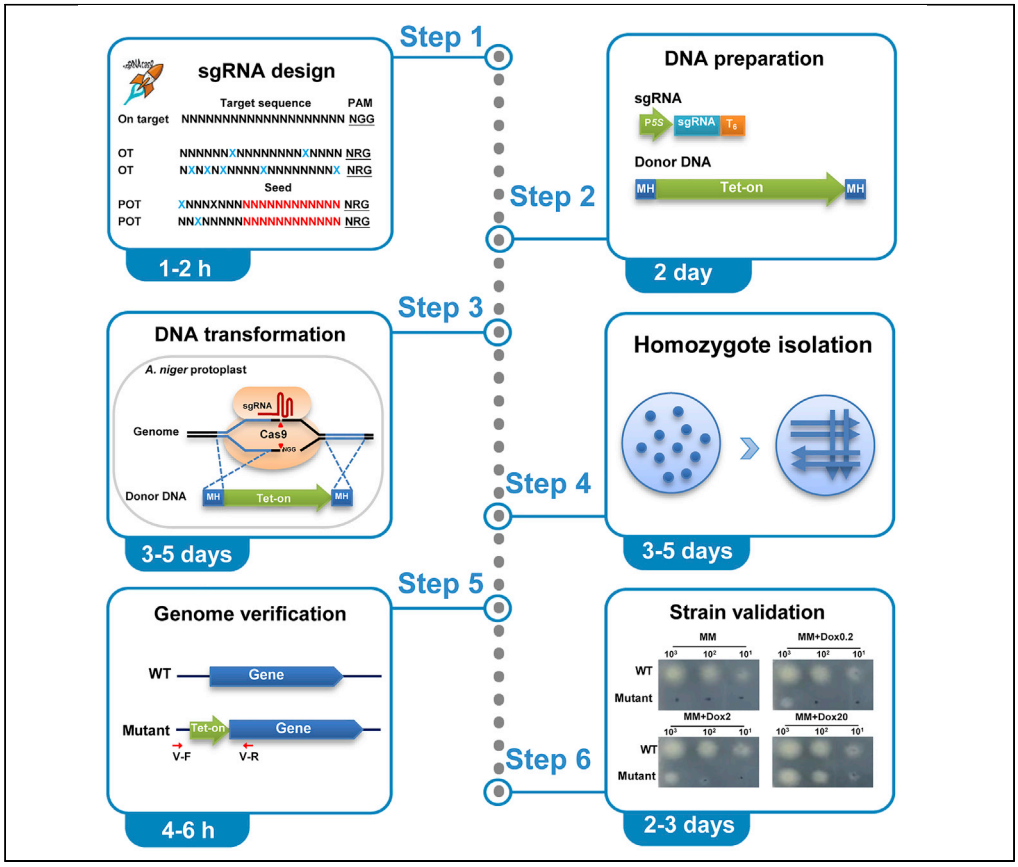
# Protocol for gene characterization in *Aspergillus niger* using 5S rRNA-CRISPR-Cas9-mediated Tet-on inducible promoter exchange




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.

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## Highlights

Protocol for highly efficient 5S rRNA-CRISPR-Cas9 genome editing in *A. niger*

Gene characterization via promoter exchange of Tet-on switch in filamentous fungi

Detailed description of DNA co-transformation by PEG-mediated protoplast transformation

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

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## 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


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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.[9–13]

### sgRNACas9 software environment setting

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[14] (sgRNAcas9_3.0.5) from website: http://www.biootools.com/software.html.

### Plasmid preparation

Timing: 1–2 h

4. Inoculate *E. coli* Trans-T1 strains containing psgRNA6.0[1] and pTC1.13[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

Timing: 4–6 h

8. Prepare the LB agar plates[15] and LB liquid media[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.[17] When necessary, add 150 μg/mL of hygromycin B for the selection marker hygromycin B phosphotransferase (*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 |

(*Continued on next page*)

*Continued*

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| $CaCl_2$ | 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 |
| $NaNO_3$ | Sigma-Aldrich | Cat#S5506 |
| $KH_2PO_4$ | Sigma-Aldrich | Cat#P0662 |
| KCl | Sigma-Aldrich | Cat#P3911 |
| $MgSO_4$ $7H_2O$ | Sigma-Aldrich | Cat#230391 |
| $ZnSO_4 \cdot 7H_2O$ | Sigma-Aldrich | Cat#Z0251 |
| $MnCl_2 \cdot 4H_2O$ | Sigma-Aldrich | Cat#M3634 |
| $CoCl_2 \cdot 6H_2O$ | Sigma-Aldrich | Cat#C8661 |
| $CuSO_4 \cdot 5H_2O$ | Sigma-Aldrich | Cat#C8027 |
| $Na_2MoO_4 \cdot 2H_2O$ | Sigma-Aldrich | Cat#M1003 |
| Uridine | Sigma-Aldrich | Cat#U3003 |
| Sobitol | Sigma-Aldrich | Cat#PHR1006 |
| Ethylenedinitrilotetraacetic acid (EDTA) | Sigma-Aldrich | Cat#M6758 |
| Lysing Enzyme | Sigma-Aldrich | Cat#L1412 |
| **Critical commercial assays** | | |
| 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 |
| BbsI | New England Biolabs | Cat#R0535S |
| FastPfu Fly PCR SuperMix | TransGen Biotech | Cat#AS231-01 |
| **Experimental models: Organisms/strains** | | |
| *Aspergillus niger*: Strain background: D353 | Shanghai Industrial Microbiology Institute Tech. Co. | SIMI: M203 |
| **Oligonucleotides** | | |
| sgRNA-pyrG-F | Zhang et al.[11] | caccGAGTAGTTCGAAGTTTCGAC |
| sgRNA-pyrG-R | Zhang et al.[11] | aaacGTCGAAACTTCGAACTACTC |
| MHi-sgRNA-pyrG-F | Zhang et al.[11] | gtatccgcgcacgtctctggatttacgaatcag ggtccaGACGTTAACTGATATTGAAG |
| MHi-sgRNA-pyrG-R | Zhang et al.[11] | ggcacgggcagtgtaggtcaatcgcgacttg gaggacatGGTGTTTAAACGGTGATGTC |
| pyrG-g-F | Zhang et al.[11] | CATGTGCAGCAGGGAATACGAG |
| pyrG-g-R2 | Zhang et al.[11] | GAGCCTTAAGTCCCTCAATGGTC |
| M13F | Zhang et al.[11] | TGTAAAACGACGGCCAGT |
| M13R | Zhang et al.[11] | CAGGAAACAGCTATGACC |
| **Recombinant DNA*** | | |
| psgRNA6.0 | Zheng et al.[1] | N/A |
| psgRNA6.15 | Zhang et al.[11] | N/A |
| pCas9-hph | Zhang et al.[11] | N/A |
| pTC1.13 | Cairns et al.[10] | N/A |
| **Software and algorithms** | | |
| sgRNACas9 | Xie et al.[14] | http://www.biootools.com |
| FungiDB | Stajich et al.[18] | https://fungidb.org |
| Java | Java Software Foundation | http://www.java.com |
| Perl | Perl Software Foundation | https://www.perl.org |
| **Other** | | |
| NanoDrop 2000 Spectrophotometer | Thermo Scientific | Cat#ND-2000 |
| Thermal Cycler | Bio-Rad | Cat#186-1096 |
| Miracloth | Calbiochem | Cat#475855 |

*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 |
| $ddH_2O$ | 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 |
| $ddH_2O$ | 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 |
| $CaCl_2$ (5 M) | 50 mM | 10 mL |
| MES buffer (200 mM, pH5.8) | 20 mM | 100 mL |
| $ddH_2O$ | 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 |
| $CaCl_2$ (5 M) | 50 mM | 10 mL |
| Tris-HCl buffer (1 M, pH7.5) | 10 mM | 10 mL |
| $ddH_2O$ | 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 |
| $CaCl_2$ (5 M) | 50 mM | 10 mL |
| Tris-HCl buffer (1 M, pH7.5) | 10 mM | 10 mL |
| $ddH_2O$ | N/A | to 1 L |
| Filter-sterilize. Store at 4°C. Stable for three months. | | |

| Protoplast lysing 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 |
| $NaNO_3$ | 3.5 M | 60.00 g |
| $KH_2PO_4$ | 350 mM | 14.97 g |
| KCl | 350 mM | 5.22 g |
| $ddH_2O$ | 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 |
| $ddH_2O$ | 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 |
| $ZnSO_4 \cdot 7H_2O$ | 0.44% | 4.40 g |
| $MnCl_2 \cdot 4H_2O$ | 0.10% | 1.01 g |
| $CoCl_2 \cdot 6H_2O$ | 0.032% | 0.32 g |
| $CuSO_4 \cdot 5H_2O$ | 0.032% | 0.32 g |
| $Na_2MoO_4 \cdot H_2O$ | 0.03% | 0.30 g |
| $CaCl_2$ | 0.11% | 1.11 g |
| $FeSO_4 \cdot 7H_2O$ | 0.10% | 1.00 g |
| $ddH_2O$ | 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 |
| $ddH_2O$ | N/A | to 1 L |

(*Continued on next page*)

| *Continued* | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| Autoclave at 121°C for 20 min and add supplements afterward before use | | |
| ASP+N (50×) | 1× | 20 mL |
| Glucose (50%) | 1% | 20 mL |
| $MgSO_4 \cdot 7H_2O$ (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 |
| $ddH_2O$ | N/A | to 1 L |
| Autoclave at 121°C for 20 min and add supplements afterward before use | | |
| ASP+N (50×) | 1× | 20 mL |
| Glucose (50%) | 1% | 20 mL |
| $MgSO_4 \cdot 7H_2O$ (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 |
| $ddH_2O$ | N/A | to 1 L |
| Autoclave at 121°C for 20 min and add supplements afterward before use | | |
| ASP+N (50×) | 1× | 20 mL |
| Glucose (50%) | 1% | 20 mL |
| $MgSO_4 \cdot 7H_2O$ (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 |
| $ddH_2O$ | N/A | to 500 mL |
| Autoclave at 115°C for 30 min and add supplements afterward before use | | |
| ASP+N (50×) | 1× | 10 mL |
| $MgSO_4 \cdot 7H_2O$ (1 M) | 2 mM | 1 mL |
| Trace elements (1000×) | 1× | 0.5 mL |
| Store at RT (25°C). Stable for one month. | | |

| MMSN-Top medium | | |
|---|---|---|
| Reagent | Final concentration | Amount |
| Sucrose | 0.95 M | 162.60 g |
| Agar | 0.6% | 3.00 g |
| $ddH_2O$ | N/A | to 500 mL |
| Autoclave at 115°C for 30 min and add supplements afterward before use | | |
| ASP+N (50×) | 1× | 10 mL |
| $MgSO_4$ $7H_2O$ (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

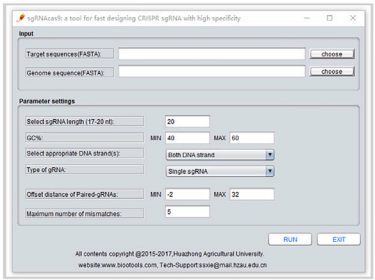
The protospacers of sgRNA determines the location, efficiency and specify of DNA cleavage by Cas9 for genome editing. Thus, we suggest designing a specific protospacer according to off-target evaluation of putative sgRNA sequences using the sgRNACas9 software.[14] sgRNAcas9 enables the fast design of 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 (https://fungidb.org/) 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/.

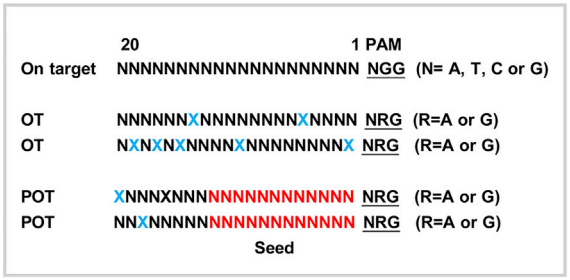
⚠ CRITICAL: Please note that the sgRNACas9 software does not allow any white space in the file name or file path.

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:

A




B




C

| sgRID | Start | End | Protospacer_sequence | Length | GC | Risk_evaluation | Total_POT | Total_OT | 0M | 1M | 2M | 3M | 4M | 5M |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| pyrG_S_63 | 821 | 843 | TCGACATTGAACATTGTCCTTGG | 23 | 0.40 | Best | 0 | 6 | 1 | 0 | 0 | 0 | 2 | 4 |
| pyrG_A_74 | 848 | 870 | TGACTGACCCTTAAATCTGAAGG | 23 | 0.40 | Best | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| pyrG_A_73 | 893 | 915 | CGTAAATCCAGAGACGTGCGCGG | 23 | 0.55 | Moderate_risk | 1 | 7 | 1 | 0 | 1 | 0 | 1 | 5 |
| pyrG_S_67 | 900 | 922 | CGTCTCTGGATTTACGAATCAGG | 23 | 0.45 | Best | 0 | 6 | 1 | 0 | 0 | 1 | 0 | 5 |
| pyrG_S_68 | 901 | 923 | GTCTCTGGATTTACGAATCAGGG | 23 | 0.40 | Best | 0 | 8 | 1 | 0 | 0 | 1 | 1 | 6 |
| pyrG_A_72 | 925 | 947 | TAGTTCGAAGTTTCGACTGGTGG | 23 | 0.45 | Best | 0 | 3 | 1 | 0 | 0 | 0 | 0 | 3 |
| pyrG_A_71 | 928 | 950 | GAGTAGTTCGAAGTTTCGACTGG | 23 | 0.45 | Best | 0 | 3 | 1 | 0 | 0 | 0 | 1 | 2 |
| pyrG_A_70 | 963 | 985 | GTTAATGCGGGAATGGAAAGAGG | 23 | 0.45 | Best | 0 | 18 | 1 | 0 | 0 | 0 | 8 | 10 |
| pyrG_A_69 | 970 | 992 | TGGAGGGGTTAATGCGGGAATGG | 23 | 0.55 | Best | 0 | 14 | 1 | 0 | 0 | 1 | 4 | 9 |
| pyrG_A_68 | 975 | 997 | GTTGATGGAGGGGTTAATGCGGG | 23 | 0.50 | Best | 0 | 30 | 1 | 0 | 0 | 0 | 12 | 18 |
| pyrG_A_67 | 976 | 998 | TGTTGATGGAGGGGTTAATGCGG | 23 | 0.45 | Low_risk | 1 | 44 | 1 | 0 | 0 | 2 | 23 | 19 |
| pyrG_A_66 | 985 | 1007 | AGGACATGGTGTTGATGGAGGGG | 23 | 0.50 | Low_risk | 1 | 49 | 1 | 0 | 0 | 1 | 17 | 31 |

D

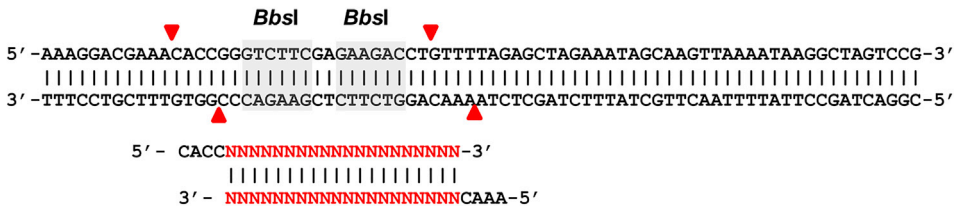



**Figure 1. sgRNA design using the sgRNACas9 software**

(A) Operation interface of sgRNACas9.

(B) The algorithms of appraisal of the off-target risk. OT, off-target, contain all types of off-target sites in the genome. POT, potential off-target, only contain off-targets that are perfect match to the 12 nt seed region of the protospacer and outer segments with mismatched bases. Blue X is represented the mismatched bases.

(C) Output example of sgRNA evaluation of the *pyrG* gene. sgRID, ID of the predicted protospacer; Start and End, start site and end site of the protospacer; GC, GC content; Risk_evaluation, evaluation of off-target risk; Total_POT, the number of off-targets with and perfect match to the 12-nt seed region and mismatch less than 5; OT, the number of off-targets with mismatch less than 5; 0 M, on target site; 1 M–5 M, off-targets with 1–5 mismatches. PyrG_A_71, located 50-bp upstream of ATG starting codon, was highlighted in bold green.

(D) sgRNA plasmid construction strategy. The *Bbs*I restriction sites in gray, partial sgRNA scaffolding sequences of psgRNA6.0 and annealing sgRNA oligos are shown.

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 *sp*Cas9 from *Streptococcus pyogenes* is NGG, but protospacers with PAM of NAG can also be cleaved with lower efficiency.[19] To choose a specific protospacer, the off-target evaluation with NAG PAM should be taken in 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[1] with *Bbs*I, and ligation with annealed overlapping complementary oligos (Figure 1D).

6. Digest psgRNA6.0 with *Bbs*I.
   a. Prepare the digestion mix in a PCR tube:

| **Digestion reaction master mix** | |
|---|---|
| Reagent | Amount |
| 10× Digestion buffer | 5 μL |
| *Bbs*I | 5 μL |
| psgRNA6.0 | 20 μL (5 μg) |
| $ddH_2O$ | 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_Plasmid 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** | | |
|---|---|---|
| Steps | 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 | |
|---|---|
| Reagent | 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) |
| $ddH_2O$ | 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 $CaCl_2$ 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 $CaCl_2$ 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

**Timing: 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 | |
|---|---|
| Reagent | 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 |
| $ddH_2O$ | 19 μL |
| Total | 50 μL |

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

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

(*Continued on next page*)

| *Continued* | | | |
|---|---|---|---|
| Steps | Temperature | Time | Cycles |
| Annealing | 55°C | 20 s | |
| Extension | 72°C | 30 s*/2 min* | |
| Final extension | 72°C | 5 min | 1 |
| Hold | 4°C | forever | |

*Extension time of the sgRNA and donor DNA fragment PCR are 30 s and 2 min, respectively.

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.[7] procedure.

16. Inoculate $2.5 \times 10^8$ 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 lysing 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 10°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.

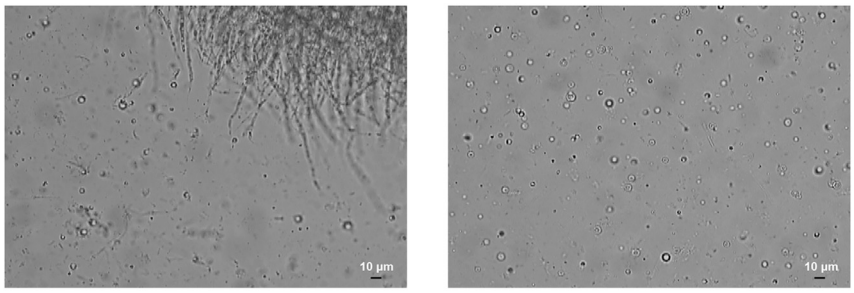



**Figure 2. Protoplasts releasing from the mycelia of *A. niger***

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.

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 supplemented 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 genotype 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 suspensions 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 Hygromycin B and 10 μg/mL Dox.
31. Repeat the monoclonal cultivation step twice for obtaining the homokaryons of each transformant, 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).

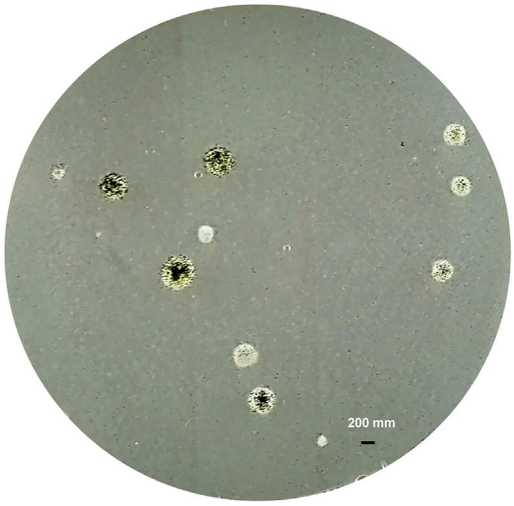



**Figure 3. An example of single colonies of subculture primary transformants of *A. niger***
Black bar represents 200 mm.

35. Genomic DNA of the homozygotes verified via diagnostic PCR and sequencing analysis with the corresponding primers (Figure 4).
    a. Prepare the PCR mix:

| PCR reaction master 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 |
| dd$H_2O$ | 7 μL |
| Total | 20 μL |

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

| PCR cycling conditions | | | |
|---|---|---|---|
| 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.

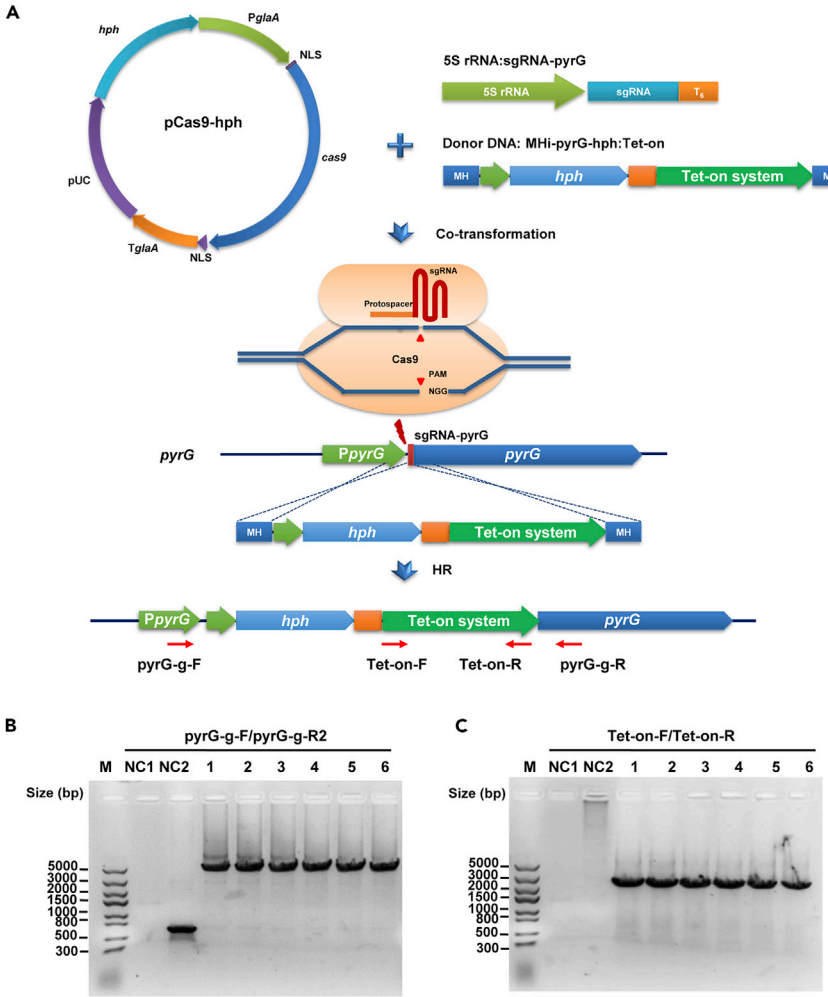



**Figure 4. Example of genomic verification of conditional expressing mutants in *A. niger***

(A) Schematic diagram of *pyrG* titratable expression mutagenesis mediated by 5S rRNA-CRISPR/Cas9 system. The donor DNA MHi-pyrG-hph:Tet-on, containing Tet-on system cassette, were co-transformed with linear sgRNA construct sgRNA-pyrG and Cas9 expression plasmid pCas9-hph into the protoplasts of *A. niger* D353. DSBs at the locus of the upstream of *pyrG* encoding sequences, were generated by the Cas9 under the guide of sgRNA-pyrG, and then were repaired by HR with the integration of donor DNA MHi-pyrG-hph:Tet-on, resulting in the replacement of *pyrG* native promoter.

(B and C) Diagnostic PCR analysis of the *pyrG* titratable expression transformants. The expected sizes of PCR products of the mutants were 5,424-bp (pyrG-g-F/pyrG-g-R2), when donor DNA MHi-pyrG-hph:Tet-on were correctly inserted at the expected loci of *pyrG*, while 1,004-bp for the parent strain D353. The expected sizes of PCR products of the Tet-on cassette in mutants were 2,575-bp (Tet-on-F/Tet-on-R). NC1, Blank control without any DNA templates; NC2, negative control with the genome of D353 as template. 1–6, the *pyrG* conditional expressing mutants. Adapted from Zhang et al.[11]

## 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).

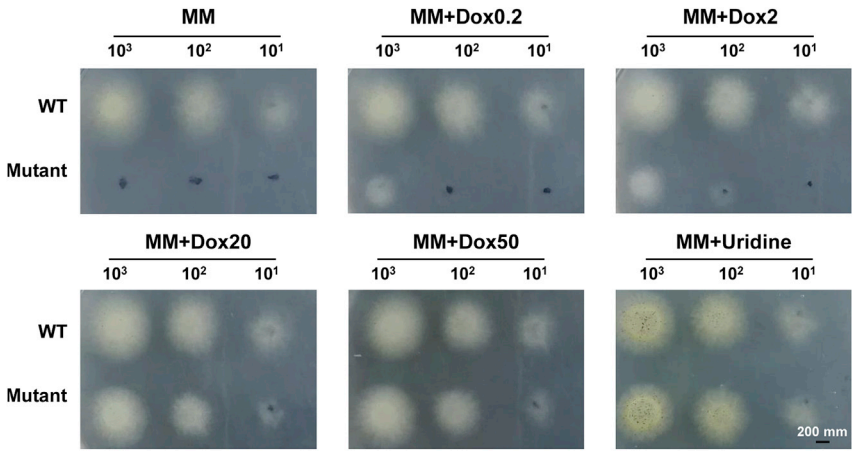



**Figure 5. An example of cell growth validation of *pyrG* conditional expression mutants on solid plates**

Spores ($1 \times 10^3$, $1 \times 10^2$, and $1 \times 10^1$) were inoculated in 2 μL volumes onto the MM supplemented with various concentrations of Dox and MM with uridine as control. Plates were incubated at 30°C in the dark for 48 h. Representative images are shown for technically triplicated experiments. Control, *A. niger* D353 as the positive controls; Mutant, the *pyrG* conditional expression mutant. Black bar represents 200 mm. Adapted from Zhang et al.[11]

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).[23]

### 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 MA70.15[7] 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.

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## 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.

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