bio-protocol

Open Access

Published: Jan 05, 2026

Creating Loss-of-Function Mutants of *Neurospora crassa* Using a Novel CRISPR/Cas9 System

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Abstract

Since its introduction, the CRISPR/Cas9 system has been used in many organisms for precise and rapid genome editing, as well as for editing multiple genes at once. This targeted mutagenesis makes it easy to analyze the function of a gene of interest (goi). The standard method for genetic manipulation of the model organism *Neurospora crassa* has been homologous recombination. It is well established and widely used to create knock-out or overexpression mutants. The recently developed CRISPR/Cas9 system is an addition to the toolkit for genetically manipulating *N. crassa*. For this protocol, a strain stably expressing the Cas9 endonuclease is required. After designing the gRNA with the online tool CHOP-CHOP, a synthetic gRNA is used to transform macroconidia via electroporation. Combining the goi-gRNA with a gRNA targeting the *csr-1* gene as a selection marker allows for easy identification of colonies with mutations at the target site of the goi, since the obtained resistance to Cyclosporin A (CsA) allows for selecting editing events. The mutation type can be detected by PCR of the edited gene region followed by Sanger sequencing. This system is fast and easy to handle, offering an attractive alternative to homologous recombination, especially for targeting multiple genes simultaneously.

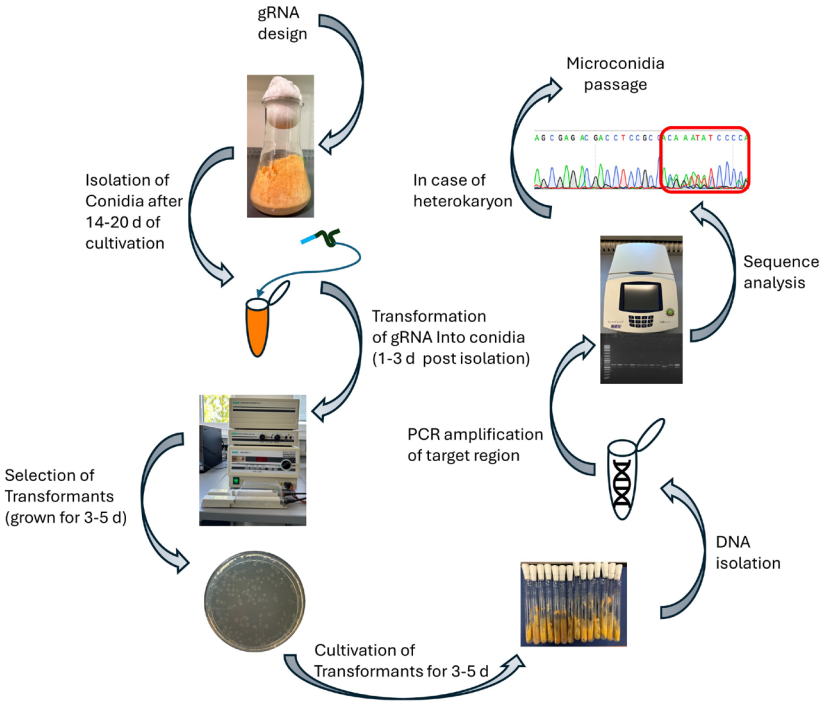
Key features

- This protocol allows the use of CRISPR/Cas9 in *Neurospora crassa* to create loss-of-function mutants.
- It can be used to create loss-of-function mutants of multiple genes in one round of transformation.
- Time-saving mutagenesis without the need for vector construction.
- In combination with *csr-1* as a selection marker, the screening for successfully edited genes of interest is reduced.

Keywords: CRISPR/Cas9, Loss-of-function mutagenesis, Genome editing, *Neurospora crassa*, Targeted mutagenesis, Electroporation, *csr-1* selection marker

This protocol is used in: Sci Rep (2024), DOI: 10.1038/s41598-024-71540-x

Graphical overview



Background

Neurospora crassa is a widely used eukaryotic model organism because of its ease of growth, genetic accessibility, and rapid life cycle. It is commonly employed in genetic research, where its genome is modified to study gene functions [1].
Traditionally, random mutagenesis methods such as UV exposure or chemicals have been used for genetic modification, which causes multiple unpredictable mutations. Identifying these mutations is time-consuming, and pinpointing the exact mutation responsible for a phenotype is difficult. Therefore, targeted mutagenesis is preferred. In *N. crassa*, homologous recombination (HR) has become the preferred method for creating targeted gene knockouts or overexpression lines. This involves transforming the organism with a vector containing homologous flanking sequences, the gene of interest, and/or a selection marker [2]. For higher efficiency, strains lacking nonhomologous end joining (NHEJ) are often used [3,4]. After transformation and the generation of homokaryotic transformants, validation is performed via PCR or Southern blot [3,5]. The whole process can take up to seven weeks, is labor-intensive, and is less efficient if NHEJ-deficient strains are not used [6]. The CRISPR/Cas9 system offers a faster and more precise alternative. It involves the Cas9 endonuclease and a guide RNA (gRNA) that directs editing to specific DNA sequences. When Cas9 induces a double-strand break, the cell repairs it either through NHEJ, which may introduce small insertions or deletions (indels), or homology-directed repair (HDR), which allows for the integration of new DNA sequences when a suitable donor DNA is available [7].
Although the CRISPR/Cas9 system has been adapted for various fungi [8], it has not yet been widely used in *N. crassa*. Another established system for *N. crassa* requires co-transforming multiple plasmids carrying Cas9, gRNA, and donor DNA, making it complex and labor-intensive [9]. In contrast, the system described here simplifies this process by integrating Cas9 directly into the genome and introducing naked synthetic gRNA via electroporation of macroconidia, eliminating the need to clone and co-transform Cas9/gRNA plasmids [10].
One concern with stable Cas9 expression is the potential for off-target mutations [11]. Studies in fungi suggest that limiting the duration of Cas9-gRNA complex presence can reduce this risk [12,13]. Even if Cas9 remains in the cells, controlling gRNA expression helps minimize off-target effects. To further reduce potential off-target mutations after successful mutagenesis, outcrossing the Cas9 sequence may be advisable. Co-transformation of multiple gRNAs enables simultaneous editing events at once. This ability allows the use of the *csr-1* gene as a selection marker in *N. crassa*. It involves co-transforming a gRNA targeting *csr-1* with a gRNA targeting the gene of interest (goi). If *csr-1* is successfully edited and becomes non-functional, the fungus exhibits resistance to Cyclosporin A (CsA). This resistance allows selection for edited strains using CsA-containing media, simplifying the identification process. Additionally, mutations in *csr-1* typically

produce homokaryons [14]. Traditionally, isolating homokaryotic strains involves time-consuming steps such as crossing, microconidia isolation, or serial transfers of macroconidia. However, these steps can be bypassed by using the CRISPR/Cas9 system with *csr-1* as a marker. Overall, this approach offers a time- and effort-saving, user-friendly method for generating loss-of-function mutants in *N. crassa*.

Materials and reagents

Biological materials

1. *Neurospora crassa* strain NcCas9SG [available at the Fungal Genetics Stock Center (FGSC); reference #27376]

Reagents

1. Sucrose (Roth, CAS number: 57-50-1)
2. Agar-agar (Roth, CAS number: 9002-18-0)
3. Citric acid (Roth, CAS number: 5949-29-1)
4. $ZnSO_4 \cdot 7H_2O$ (Merck, CAS number: 7446-20-0)
5. $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ (Merck, CAS number: 7783-85-9)
6. $CuSO_4 \cdot 5H_2O$ (Merck, CAS number: 7758-99-8)
7. $MnSO_4 \cdot H_2O$ (Roth, CAS number: 10034-96-5)
8. H_3BO_3 (Roth, CAS number: 10043-35-3)
9. $Na_2MoO_4 \cdot 2H_2O$ (Merck, CAS number: 10102-40-6)
10. Chloroform (Roth, CAS number: 67-66-3)
11. Na_3-Citrate$\cdot 2H_2O$ (Roth, CAS number: 6132-04-3)
12. KH_2PO_4 (Roth, CAS number: 7778-77-0)
13. NH_4NO_3 (Roth, CAS number: 6484-52-2)
14. $MgSO_4 \cdot 7H_2O$ (Merck, CAS number: 10034-99-8)
15. $CaCl_2 \cdot 2H_2O$ (Roth, CAS number: 10035-04-8)
16. Biotin (Roth, CAS number: 58-85-5)
17. TRIS (Roth, CAS number: 77-86-1)
18. NaCl (Roth, CAS number: 7647-14-5)
19. KCl (Roth, CAS number: 7447-40-7)
20. EtOH p.a. (Roth, CAS number: 64-15-7)
21. EDTA (Roth, CAS number: 6381-92-6)
22. Na-iodoacetate (Merck, CAS number: 305-53-3)
23. KNO_3 (Roth, CAS number: 7757-79-1)
24. K_2HPO_4 (Roth, CAS number: 7758-11-4)
25. KOH (Roth, CAS number: 1310-58-3)
26. Cyclosporin A (biomol, catalog number: AG-CN2-0079-M100)
27. Sorbitol (Roth, CAS number: 50-70-4)
28. Tween 80 (Roth, CAS number: 9005-65-6)
29. Alt-R™ CRISPR-Cas9 tracrRNA (20 nmol) (IDT, catalog number: 1072533)
30. Duplex buffer (IDT, catalog number: 11-01-03-01)

Solutions

1. 50× Vogel's solution (see Recipes)
2. Trace elements solution (TES) (see Recipes)
3. Vogel's minimal medium + sucrose (VMM+S) (see Recipes)
4. Vogel's minimal medium + sorbose-fructose-glucose + cyclosporin A (VMM+SGF+CsA) (see Recipes)
5. Sorbose-fructose-glucose solution (SGF) (see Recipes)
6. Top agar (see Recipes)
7. TPS buffer (see Recipes)
8. MKM medium (see Recipes)

9. 2× Synthetic crossing (SC) solution (see Recipes)
11. 1 M sorbitol + 0.25% Tween 80 (see Recipes)

Recipes

1. 50× Vogel's solution

Reagent	Final concentration	Quantity or volume
Na_3-Citrate·$2H_2O$	510 mM	75 g
KH_2PO_4	1.84 M	125 g
NH_4NO_3	1.25 M	50 g
$MgSO_4·7H_2O$	40.6 mM	5 g
$CaCl_2·2H_2O$*	34 mM	2.5 g
TES	1% (v/v)	5 mL
Biotin	0.1 mM	12.5 mg
Chloroform**	0.2% (v/v)	1 mL
Autoclaved ddH_2O	n/a	Add up to 500 mL
Total	n/a	500 mL

*Before adding, dissolve in 50 mL of ddH_2O.
**Chloroform is added as a preservative and is optional.
Store Vogel's solution in the dark at room temperature (RT).

2. Trace element solution (TES)

Reagent	Final concentration	Quantity or volume
Citric acid	5% (w/v)	5 g
$ZnSO_4·7H_2O$	5% (w/v)	5 g
$Fe(NH_4)_2(SO_4)_2·6H_2O$	1% (w/v)	1 g
$CuSO_4·5H_2O$	0.25% (w/v)	250 mg
$MnSO_4·H_2O$	0.05% (w/v)	50 mg
H_3BO_3	0.05% (w/v)	50 mg
$Na_2MoO_4·2H_2O$	0.05% (w/v)	50 mg
Chloroform*	0.2% (v/v)	0.2 mL
Autoclaved ddH_2O	n/a	Add up to 100 mL H_2O
Total	n/a	100 mL

*Chloroform is added as a preservative and is optional.
Store TES at 4 °C.

3. Vogel's minimal medium + sucrose (VMM+S)

Reagent	Final concentration	Quantity or volume
50× Vogel's solution	1×	10 mL
Sucrose	20 g/L	10 g
Agar-agar	2% (w/v)	10 g
ddH_2O	n/a	Add up to 500 mL
Total	n/a	500 mL

The pH of the minimal medium is about 5.8. No adjustment is necessary.
Autoclave at 112 °C. Pour into flasks or slant agar tubes on a sterile workbench. Allow to cool and set at RT. Store at 4 °C until needed.

4. Vogel's minimal medium + sorbose-fructose-glucose + cyclosporin A (VMM+SGF+CsA)

Reagent	Final concentration	Quantity or volume
50× Vogel's solution	1×	10 mL
Agar-agar	2% (w/v)	10 g
ddH_2O	n/a	Add up to 475 mL
SGF*	5% (v/v)	25 mL
Cyclosporin A (5 mg/mL)*	5 µg/mL	500 µL

Total	n/a	500 mL

*Add after autoclaving.

Autoclave at 121 °C, mix, and pour into 92 mm × 16 mm Petri dishes in a sterile workbench. Allow to cool and set at RT. Store at 4 °C until needed.

5. Sorbose-fructose-glucose solution (SGF)

Reagent	Final concentration	Quantity or volume
Sorbose	20% (w/v)	20 g
Fructose	0.5% (w/v)	0.5 g
Glucose	0.5% (w/v)	0.5 g
ddH_2O	n/a	Add up to 100 mL
Total	n/a	100 mL

Autoclave at 112 °C. Let it cool down at RT. Store at 4 °C until needed.

6. Top agar

Reagent	Final concentration	Quantity or volume
50× Vogel's solution	1×	2 mL
Agarose	1% (w/v)	1 g
ddH_2O	n/a	Add up to 95 mL
SGF*	5% (v/v)	5 mL
Cyclosporin A (5 mg/mL)*	5 µg/mL	100 µL
Total	n/a	100 mL

*Add after autoclaving.

Autoclave at 121 °C, add SGF, and keep at 45 °C to avoid solidification.

7. TPS buffer

Reagent	Final concentration	Quantity or volume
1 M Tris-HCl, pH 8.0	100 mM	10 mL
0.5 M EDTA, pH 8.0	10 mM	2 mL
KCl	1 M	7.55 g
ddH_2O	n/a	Add up to 100 mL
Total	n/a	100 mL

Autoclave at 121 °C or filter sterilize. Store at RT.

8. MKM medium

Reagent	Final concentration	Quantity or volume
Sucrose	0.5% (w/v)	2 g
2× SC solution (Recipe 9)	0.1×	10 mL
100 mM Na-Iodoacetate solution*	1 mM	2 mL
Agar-agar	2% (w/v)	4 g
ddH_2O	n/a	Add up to 198 mL
Total	n/a	200 mL

*Add after autoclaving.

Autoclave at 112 °C. After cooling to 45 °C, add the Na-Iodoacetate and pour into 92 mm × 16 mm Petri dishes in a sterile workbench. Allow to cool and set at RT. Store at 4 °C until needed.

9. 2× Synthetic crossing (SC) solution

Reagent	Final concentration	Quantity or volume
KNO_3	19.8 mM	400 mg
K_2HPO_4	8 mM	280 mg
KH_2PO_4	7.34 mM	200 mg
$MgSO_4 \cdot 7H_2O$	8 mM	400 mg
NaCl	3.4 mM	40 mg
$CaCl_2 \cdot 2H_2O$	1.4 mM	40 mg

Biotin	0.04 mM	2 mg
TES	0.02% (v/v)	40 µL
Chloroform*	0.5% (v/v)	1 mL
Autoclaved ddH_2O	n/a	Add up to 200 mL
Total	n/a	200 mL

*Chloroform is added as a preservative and is optional.
Store at 4 °C in the dark.

10. 1 M Sorbitol + 0.25 % Tween 80

Reagent	Final concentration	Quantity or volume
Sorbitol	1 M	18,2 g
Tween 80	0.25 % (v/v)	0.25 mL
ddH_2O	n/a	Add up to 100 mL
Total	n/a	100 mL

Autoclave at 112 °C. Store at RT.

Laboratory supplies

1. Erlenmeyer Flask, wide neck, 200 mL (Roth, catalog number: X737.1)
2. 1.5 mL Eppendorf tube (Sarstedt, catalog number: 72.690.001)
3. 2 mL Eppendorf tube (Sarstedt, catalog number: 72.695.500)
4. 50 mL Sarstedt tube (Sarstedt, catalog number: 62.547.254)
5. 1,000 µL pipette tips (Sarstedt, catalog number: 70.3050.020)
6. 200 µL pipette tips (Sarstedt, catalog number: 70.3030.020)
7. 10 µL pipette tips (Sarstedt, catalog number: 70.3010)
8. Petri dish, 92 mm × 16 mm (Sarstedt, catalog number: 82.1473.001)
9. Electroporation cuvette, 0.2 cm (Roth, catalog number: PP39.1)
10. Gauze (from medical supplier)
11. Funnel 65 mm (Roth, catalog number: HY46.1)
12. Parafilm M (Roth, catalog number: CNP8.1)
13. Glass test tube with beaded rim, 180 mm × 18 mm (Roth, catalog number: C209.1)
14. Glass test tube with no rim, 130 mm × 16 mm (Roth, catalog number: C188.1)
15. Cellophane made from viscose (any online supplier)
16. Whatman paper 0.35 mm thick (Roth, catalog number: CL66.1)

Equipment

1. PCR machine (e.g., SensoQuest Labcycler, biolab, catalog number: 11-011-103-XXX)
2. Thoma counting chamber (Roth, catalog number: T732.1)
3. Centrifuge (e.g., Beckmann Coulter, model: Allegra X-30R)
4. Electroporation device (e.g., Bio-Rad, model: Genepulser II)
5. Pipettes (Eppendorf)
6. Lancet needle (Roth, catalog number: KY00.1)
7. Inoculation loop (Roth, catalog number: KL99.1)
8. Heating block (e.g., Eppendorf, model: Thermomixer comfort)
9. NanoDrop

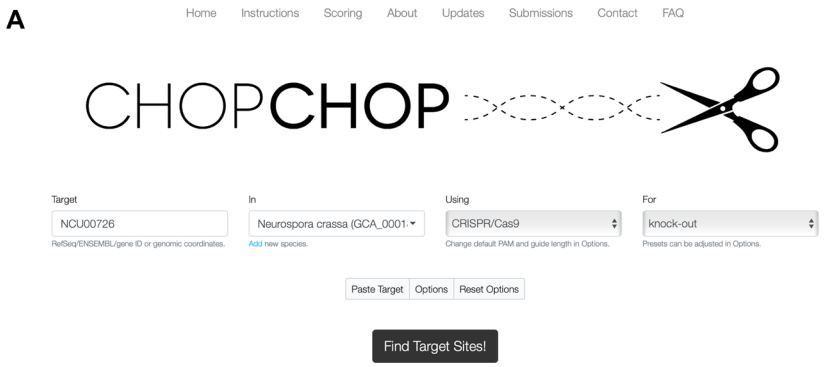
Software and datasets

1. CHOP-CHOP tool (https://chopchop.cbu.uib.no/) [15]; online tool used for gRNA design

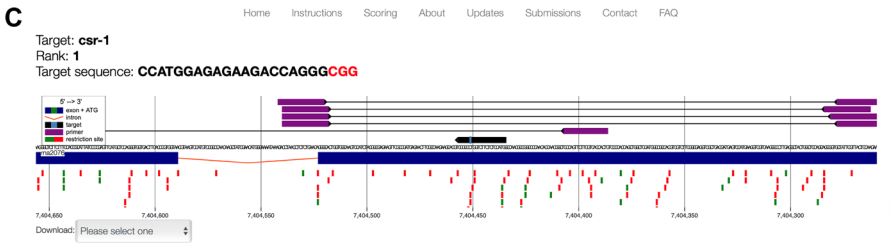
Procedure

A. Designing gRNA and duplex formation

1. Go to the website of the CHOP-CHOP tool (https://chopchop.cbu.uib.no/) for the design of the crRNA (see Figure 1A).



Rank	Target sequence	Genomic location	Strand	GC content (%)	Self-complementarity	MM0	MM1	MM2	MM3	Efficiency
1	CCATGGAGAGAAGACCAGGGCGG	CM002236.1:7404436	+	60	0	0	0	0	0	76.46
2	AAGCCCACCATTGTCGACTGCGG	CM002236.1:7404240	-	55	0	0	0	0	0	69.39
3	AGACCTAACCTCTCTGAACAGGG	CM002236.1:7404522	-	45	0	0	0	0	0	68.96
4	TTAAGGACAGAGAAATGGCGGGG	CM002236.1:7405300	+	45	1	0	0	0	0	69.09
5	GAGAGATGACGAGAAAGTCGAGG	CM002236.1:7405257	+	50	0	0	0	0	0	67.92
6	GCGCTCTCTAAATTCCCTTGTGG	CM002236.1:7404218	-	50	0	0	0	0	0	67.78
7	ATCCCCGAGTTCATGCTCCAGGG	CM002236.1:7404612	-	55	0	0	0	0	0	65.77
8	AAGCCCACCAGCGAAATCAAGGG	CM002236.1:7405029	-	50	0	0	0	0	0	65.43



Pair	Left primer coordinates	Left primer	Left primer Tm	Left primer off-targets	Right primer coordinates	Right primer	Right primer Tm	Right primer off-targets	Pair off-targets	Product size
1	CM002236.1:7404632-7404654	CTCTTCTTTCCACCGCATTATC	60.1	0	CM002236.1:7404387-7404408	ACAGTGGTGACGAAGAACTGG	60.2	0	0	267
2	CM002236.1:7404519-7404541	CTAACCTCTCTGAACAGGGCAC	60.3	0	CM002236.1:7404257-7404279	GGGCTTCTTGGAGTAACGAATA	59.6	0	0	284
3	CM002236.1:7404519-7404541	CTAACCTCTCTGAACAGGGCAC	60.3	0	CM002236.1:7404259-7404282	GCTTCTTGGAGTAACGAATAGCA	59.9	0	0	282
4	CM002236.1:7404519-7404541	CTAACCTCTCTGAACAGGGCAC	60.3	0	CM002236.1:7404263-7404285	CTTGGAGTAACGAATAGCACCG	61.0	0	0	278
5	CM002236.1:7404521-7404543	ACCTAACCTCTCTGAACAGGGC	61.0	0	CM002236.1:7404257-7404279	GGGCTTCTTGGAGTAACGAATA	59.6	0	0	286

Off-targets		
Location	Number of mismatches	Sequence (including mismatches)
There are no off-targets.		

Shen et al. 2018 predictions of repair profile - statistics	
Reference sequence	GTGACGAAGAACTGGGAGCCGTTGGTGTTGGGGCCGGCGTTGGCCATGGAGAGAAGACCA<>GGGCGGACGTGCTTCTTGGCGAAGTTCTCATCGGCGAACTTCTCGCCGTAGATGGACTTG
Frameshift frequency	82.34
Precision score	0.45
Frame +0 frequency	17.66
Frame +1 frequency	44.43
Frame +2 frequency	37.90
1-bp ins frequency	20.14
Highest del frequency	14.89
Highest ins frequency	18.94
Highest outcome frequency	18.94
Microhomology deletion frequency	56.01
Microhomology-less deletion frequency	23.85

Figure 1. Example for designing a crRNA using the online tool CHOP-CHOP for the *csr-1* gene of *Neurospora crassa*. (A) Define the parameters for the design of the crRNA. Define the target (*csr-1*), the organism (*N. crassa*), the endonuclease

(Cas9), and the purpose of the editing (knock-out). (B) The result page, showing possible target sequences with the PAM in red, the genomic location as a number and on the gene (green: good; yellow: moderate; red: not good), the targeted strand (+/-), the GC content, self-complementarity, number of off-targets (MM0-MM3; 0 = no mismatches, 1 = one mismatch, and so on), and efficiency. (C) Detailed results for a chosen target sequence with information on the predicted editing site (light blue rectangle), oligonucleotides (purple) that can be used for the PCR analysis of the target site after editing, information on off-target sites, and prediction of possible outcomes according to Shen et al. [16].

2. Enter the RefSeq/ENSEMBL/gene ID/genomic coordinates or FASTA sequence of the gene to be edited in the *Target* field.
3. Choose the *Neurospora crassa* genome in the *In* field.
4. Choose *CRISPR/Cas9* in the *Using* field.
5. In the *For* field, choose *knock-out.*
6. Then, click on *Find Target Sites!*
7. Various target sequences will be visualized with their location in the gene sequence as green (good), yellow (moderate), and red (bad) bars (see Figure 1B).

8. For a loss-of-function mutation, make sure that the gRNA binding site is:
a. Not too close to the start codon (at least 50 bp downstream) to avoid usage of alternative start codons.
b. Not too close to the stop codon (at least 300 bp upstream) to avoid generating a truncated but still functional protein.
c. Inside an exon.
Note: For detailed instructions on gRNA design with CHOP-CHOP, see Labun et al. [17].

9. In addition to the visualization, there is a table summarizing the target sequence features (see Figure 1B). Make sure the following parameters are given for your chosen sequence:
a. GC content of 40%–70%.
b. No self-complementarity.
c. No off-target effects (the MM0-MM4 values show the number of off-targets).

10. Select your desired gRNA sequence. This will lead to the individual results page with more information on the selected target sequence (see Figure 1C).
Critical: The sequence result also contains the PAM sequence. Keep in mind that the gRNA should not include the PAM.
Note: This protocol describes the use of crRNA and tracrRNA ordered from IDT Integrated DNA Technologies (https://eu.idtdna.com/pages/products/crispr-genome-editing/alt-r-crispr-cas9-system) to synthesize the gRNA. If you decide to use a different method for the synthesis of your gRNA, follow the respective instructions.

11. Order lyophilized Alt-R™ CRISPR-Cas9 crRNA (10 nmol) and Alt-R™ CRISPR-Cas9 tracrRNA (20 nmol) (IDT, catalog number: 1072533) at IDT.
Note: Ordering crRNA and tracrRNA separately is economically preferred over ordering a gRNA for each gene of interest. The tracrRNA can be used for several crRNAs, making it cheaper when editing more than one gene.
12. Reconstitute the crRNA and tracrRNA to 200 µM with IDT duplex buffer. In case of 10 nmol crRNA, add 50 µL of IDT duplex buffer. For 20 nmol tracrRNA, add 100 µL of IDT duplex buffer.
13. Mix the two RNAs in an equimolar ratio, e.g., 10 µL of each RNA, and incubate for 5 min at 95 °C in a heating block.
14. Then, let it cool down to room temperature to form the crRNA:tracrRNA duplex.
Pause point: Either use the gRNA directly for transformation or store it at -20 °C, where it remains stable for up to two years.

B. Isolation of macroconidia

1. Prepare a 200 mL flask with 50 mL of VMM+S agar and close it with a sterile cotton plug.
2. Inoculate the flask with some macroconidia of the *N. crassa* strain NcCas9SG and incubate for 14–20 days at 25 °C in a light/dark cycle (16 h light, 8 h dark) in a climate chamber.
3. After incubation, carefully pour 40 mL of 1 M sorbitol + 0.25% (v/v) Tween 80 into the flask. Let the solution run down the side of the flask to avoid stirring up the conidia.
4. Tightly seal the flask with parafilm and shake vigorously until all conidia are suspended.
5. Prepare a 50 mL Sarstedt tube with a sterile funnel lined with four layers of sterile gauze on top.

6. Pour the conidia suspension via the funnel and through the gauze into the Sarstedt tube to remove hyphae and agar from the suspension.
7. Centrifuge the suspension at 4,255× *g* for 5 min, either in a swing-out or fixed-angle rotor.
8. Remove the supernatant.
9. To wash the Tween out, fully resuspend the pellet in 50 mL of 1 M sorbitol by gently shaking the tube.
10. Centrifuge the suspension at 4,255× *g* for 5 min, either in a swing-out or fixed-angle rotor, and remove the supernatant.
11. Repeat steps B8–10 twice (three washing steps in total).
12. After the third washing step, resuspend the conidia in 500 µL of 1 M sorbitol and transfer them into a 2 mL Eppendorf tube.
13. Dilute the conidia 1:100 and determine their concentration by using a Thoma counting chamber. Count the conidia of four diagonally arranged big squares. To calculate the amount of conidia for 1 mL, use this formula: $n/16 \times 10^6$ spores (see Figure 2).
14. Adjust the concentration to 2.5×10^9 spores/mL.
Pause point: The spores stay viable for up to three days when kept at 4 °C.

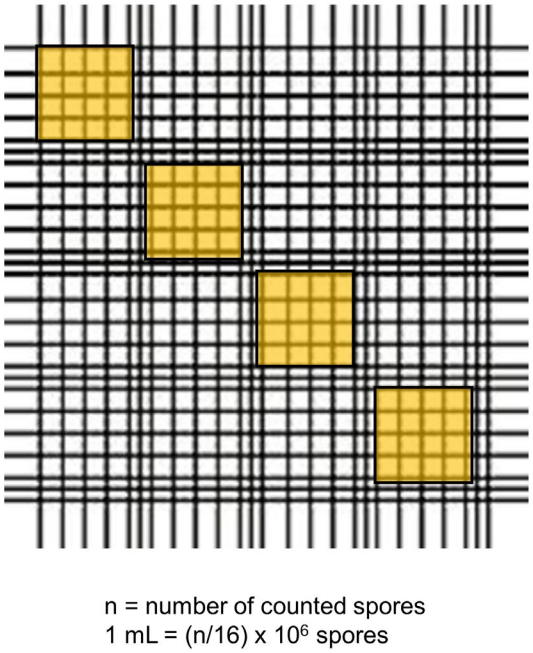


Figure 2. Scheme of the Thoma counting chamber. In yellow are the squares that should be counted. Then, use the given formula to determine the amount of conidia per milliliter.

C. Transformation of macroconidia with gRNA

1. Prechill the 0.2 cm electroporation cuvettes on ice.
2. Transfer 40 µL of the 2.5×10^9 spores/mL into a 1.5 mL Eppendorf cup for each transformation assay.
3. Mix the 40 µL suspension with 2 µL of 100 µM gRNA targeting the *csr-1* gene (as a selection marker) and 2 µL of 100 µM target gRNA.
4. As a control, mix 40 µL of suspension with 2 µL of sterile H_2O.
5. All samples need to be incubated on ice for 5 min. Then, transfer the samples into the chilled electroporation cuvette.
6. Use the following conditions for electroporation with the Bio-Rad Gene Pulser: 1.5 kV, 25 µF, and 600 Ω.
7. After the pulse, add 1 mL of 1 M sorbitol (prechilled) into the cuvette and mix carefully with a pipette. Leave the cuvettes at RT for at least 10 min before plating on VMM+SGF+CsA.
8. For plating, prepare top agar in glass test tubes (8 mL/tube) and keep it at 45 °C in a water bath.
Critical: The conidia are not viable at temperatures >45 °C.
9. Dilute the electroporated suspension 1:100 with 1 M sorbitol before plating.
10. Mix 200 µL of the dilution with 8 mL of top agar in the test tube by vortexing, then pour the top agar containing the macroconidia on the VMM+SGF+CsA plate.

11. Incubate the plate for 3–5 days at 25 °C under a light/dark cycle in the climate chamber until colonies form (see Figure 3). The average editing efficiency of this protocol lies between 7.35% and 11.89% (see [10]).
12. Transfer the colonies to slant agar tubes containing VMM+S medium and incubate in the climate chamber (25 °C, light/dark cycle of 8/16 h) for 3–5 days until macroconidia form.

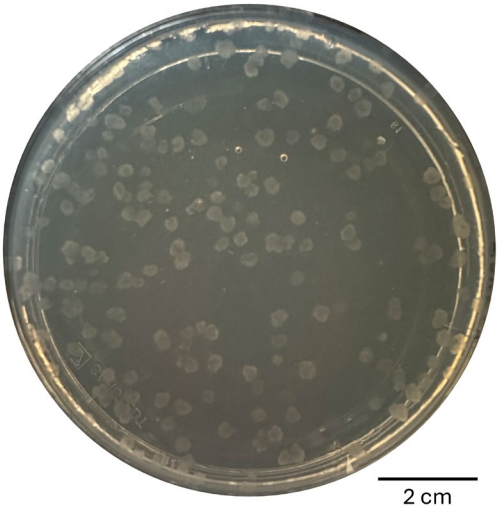


Figure 3. Example of colonies of *N. crassa* after transformation, plated on VMM+SGF+CsA. Cut out single colonies and transfer them onto slant agar tubes for further analysis.

D. Screening of editing event

1. Isolate DNA from the putative transformants using the quick method described here.
2. Collect conidia from the slant agar tubes with a sterile inoculation loop and transfer the conidia to a 1.5 mL Eppendorf cup filled with 160 µL of TPS buffer.
3. Incubate for 10 min in a heating block set to 99 °C. Alternatively, incubation for 10 min in a boiling water bath is also possible.
4. After incubation, transfer the samples immediately to ice.
5. Keep them on ice for at least 10 min.
6. Centrifuge the samples at 15,000× *g* for 5 min at RT.
7. Transfer 140 µL of the supernatant into a new 1.5 mL Eppendorf cup filled with 100 µL of sterile ddH_2O, 6 µL of 4 M NaCl, and 480 µL of EtOH p.a.
8. Incubate at -20 °C for at least 20 min to precipitate the DNA.
Note: The precipitation of the DNA can be prolonged overnight.
9. Centrifuge the samples at 15,000× *g* for 15 min at 4 °C and discard the supernatant.
10. Wash the pellet with 800 µL of 70% EtOH by centrifuging at 15,000× *g* for 20 min at 4 °C.
11. Discard the supernatant and resuspend the pellet in 35 µL of 5 mM Tris-HCl, pH 8.0.
12. Measure the DNA concentration with a NanoDrop and bring the concentration to 15 ng/µL.
13. Use 15 ng of DNA for a PCR with your favored polymerase using oligonucleotides flanking the mutation site.
Note: Either use oligonucleotides designed by you or use one of the pairs recommended by CHOP-CHOP when designing the gRNA (see Figure 1C).
14. Sequence the obtained PCR product and check for mutations at the desired mutation site.
Note: In case of heterokaryons, proceed with microconidia passage (see section E) to obtain homokaryons.

E. Microconidia passage

1. Prepare MKM plates and cut the cellophane into circles that fit exactly inside the Petri dish (92 mm in diameter). Boil the cellophane in 800 mL of 1% KOH for 5 min to remove soluble impurities.
2. Cut Whatman paper (30 × 30 cm) and soak it in ddH_2O. Lay the boiled cellophane circles on the lower part of the Whatman paper next to each other and fold the paper over the cellophane so that it is wrapped in the paper. Put the package in a 1 L

beaker with 50 mL of ddH_2O and autoclave at 121 °C for 20 min.
3. Layer the autoclaved cellophane circle on top of the MKM medium using sterile forceps. Work in a sterile workbench. Make sure to evenly distribute the cellophane. Make sure that no air is trapped between the cellophane and the agar.
Critical: Make sure that the cellophane stays humid throughout the procedure. Otherwise, the handling is more difficult.
4. Now, take macroconidia from a slant agar culture with an inoculation loop and transfer them into a 1.5 mL Eppendorf cup filled with 50 µL of sterile ddH_2O.
5. Take 1.5 µL of the conidia suspension. Using the pipette tip, pierce a hole in the middle of the cellophane circle layered on top of the MKM medium and transfer the suspension between the agar and the cellophane.
6. Wrap parafilm around the Petri dish to keep the inside humid and incubate the plates for 7–12 days inside a climate chamber at 25 °C in a light/dark cycle (16 h light, 8 h dark).
7. After this time, remove the cellophane carefully from the plates and keep the plates one more day in the climate chamber at 25 °C in a light/dark cycle (16 h light, 8 h dark).
8. Now, use an inoculation loop and dip it in sterile ddH_2O so that the inner circle of the loop is filled with water. Swipe across the grown mycelia on the plate. The microconidia will be collected inside the water.
9. Transfer the microconidia on VMM+SGF plates to obtain single homokaryotic colonies.

Data analysis

The typical mutation frequency depends on the gRNA used for editing and the accessibility of the target sequence within the genomic DNA. When designing the gRNA with the CHOP-CHOP tool, mutation frequencies are predicted from Shen et al. [16]. Table 1 shows experimental mutation frequencies for two different gRNAs targeting the *csr-1* gene using the experimental data from [10]. Target site 1 refers to a gRNA targeting the minus strand in the third exon of *csr-1,* and target site 2 refers to a gRNA targeting the plus strand of the fourth exon of *csr-1*.

Table 1. Example of different mutation frequencies for two gRNAs targeting the *csr-1* gene from [10].

Type of mutation	Frequency for target site 1	Frequency for target site 2
1 bp insertion	50%	30.18%
2 bp insertion	0%	7.5%
>2 bp insertion	0%	1.9%
1 bp deletion	12.5%	22.6%
2 bp deletion	18.75%	20.8%
>2 bp deletion	18.75%	17%

Usually, 20 colonies were screened to find a successful edit of the gene of interest.

Validation of protocol

This protocol has been used and validated in the following research article:

- Grüttner and Kempken. [10] A user-friendly CRISPR/Cas9 system for mutagenesis of *Neurospora crassa*. *Scientific Reports*. 14(1): 20469. https://doi.org/10.1038/s41598-024-71540-x

General notes and troubleshooting

Troubleshooting

Problem 1: Low transformation efficiency.
Possible causes: Condition of macroconidia (might be too old), or the top agar was too hot.
Solution 1: Make sure the macroconidia are not too old. After isolation of the macroconidia, the transformation efficiency is reduced drastically if stored for longer than three days. Best use them right after isolation.
Solution 2: Isolate macroconidia no later than 20 days after inoculation of the Erlenmeyer flask.
Solution 3: Make sure the temperature of the top agar for plating after the electroporation is not above 45 °C.

Problem 2: No editing event can be detected in the sequencing results after PCR of the putatively edited region.
Possible cause: The used target sequence for the gRNA was not optimal.
Solution 1: Follow the detailed instructions for gRNA design in Labun et al. [17] and/or the CHOP-CHOP website.
Solution 2: Make sure that the PAM sequence is not included in the sequence of the crRNA.
Solution 3: Use more gRNA for the electroporation.

Acknowledgments

Authors' contribution: Conceptualization, S.G.; Investigation, S.G.; Writing—Original Draft, S.G.; Writing—Review & Editing, S.G.; Supervision, S.G.
There are no funding sources that supported the work. This protocol is based on a previously published article by Grüttner and Kempken [10].

Competing interests

The author declares no conflicts of interest.

Received: October 15, 2025; Accepted: December 07, 2025; Available online: December 15, 2025; Published: January 05, 2026

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