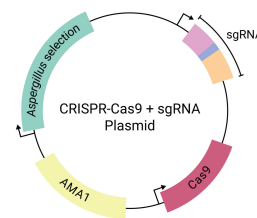


Oct 10, 2024

Genetic engineering of *A. nidulans* using CRISPR-Cas9

DOI

<https://dx.doi.org/10.17504/protocols.io.6qpvr3z93vmk/v1>



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DOI: <https://dx.doi.org/10.17504/protocols.io.6qpvr3z93vmk/v1>

Protocol Citation: Domenico Modaffari, Edward Wallace, Kenneth Sawin 2024. Genetic engineering of *A. nidulans* using CRISPR-Cas9. [protocols.io https://dx.doi.org/10.17504/protocols.io.6qpvr3z93vmk/v1](https://dx.doi.org/10.17504/protocols.io.6qpvr3z93vmk/v1)

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Protocol status: Working

We use this protocol and it's working

Created: January 14, 2024

Last Modified: October 10, 2024

Protocol Integer ID: 93510

Keywords: aspergillus, CRISPR, engineering of aspergillus nidulan, using crispr, genetic engineering, aspergillus nidulan, crispr, cas9 this protocol, cas9, single plasmid, strain, step golden gate reaction

Funders Acknowledgements:

Wellcome Trust

Grant ID: 218470

Wellcome Trust

Grant ID: 208779

Wellcome Trust

Grant ID: 210659

Wellcome Trust

Grant ID: 203149

Wellcome Trust

Grant ID: 226791

Abstract

This protocol described the engineering of *Aspergillus nidulans* strains using CRISPR-Cas9. The system is built into a single plasmid in a one-step Golden Gate reaction.



Materials

CRISPR-Cas9 vector plasmid (one or more of the following):

- pDM026 (Addgene #216808), *pyrG* selection.
- pDM028 (Addgene #216809), *hph* selection.
- pDM030 (Addgene #216810), *ble* selection.
- pDM068 (Addgene #216811), *NAT* selection.

For plasmid construction:

 PaqCI **New England Biolabs Catalog #R0745S**

 T4 DNA Ligase - 20,000 units **New England Biolabs Catalog #M0202S**

For *E. coli* transformation and plasmid purification (as required by your preferred *E. coli* transformation method):

- LB + Ampicillin (100 µg/mL) agar plates.
- LB + Ampicillin (100 µg/mL) liquid media.
- Mini-prep kit.
- Primer oDM154 for plasmid verification (5' - TTGAGCAAACCTCTGATCGCCTG - 3').

For production of repair template:

- High-fidelity polymerase.
- PCR cleanup kit.

For *A. nidulans* transformation as in **Oakley et al., 2012**:

- $\Delta nkuA$ *A. nidulans* strain.
- YG medium + supplements as required.
- KCl citrate solution, pH 5.8.
- 0.6 M KCl solution.
- 0.6 M KCl, 50 mM CaCl_2 solution.
- Polyethylene glycol (PEG) solution.
- 1.2 M Sucrose solution, cooled to 4°C.
- VinoTaste Pro (Novozymes) or Extralyse (Laffort).
- 0.22 µm low protein binding syringe filter unit.
- Miracloth (Merck Millipore, Catalog No. 475855).
- 1 M Sucrose selection plates (150 µg/mL nourseothricin for NAT selection).
- Eppendorf Protein LoBind® 1.5 mL Tubes (Catalog No. 022431081).
- Eppendorf Protein LoBind® 15 mL Conical Tubes (Catalog No. 0030122216)
- 50 mL Conical tubes.

Equipment:

- Shaking incubator (30°C and 37°C).
- Static incubators (30°C and 37°C).



- Thermocycler.
- Refrigerated microcentrifuge.
- Refrigerated centrifuge, with adapters for 50 mL and 15 mL conical tubes.
- *Optional:* tetrad dissection microscope (e.g. Singer MSM 400).

Protocol materials

⊗ LongAmp Taq DNA Polymerase - 500 units **New England Biolabs Catalog #M0323S**

⊗ Platinum™ SuperFi II DNA Polymerase **Invitrogen - Thermo Fisher Catalog #12361010**

⊗ PaqCI **New England Biolabs Catalog #R0745S**

⊗ T4 DNA Ligase - 20,000 units **New England Biolabs Catalog #M0202S**

Design sgRNA spacer and order oligos

- 1 Design your genetic modification and your 20 bp single-guideRNA (sgRNA) spacer for Cas9. 

Necessary criteria:

- The protospacer sequence within the genome must be directly 5' of a Protospacer Adjacent Motif (PAM), which for Cas9 is 'NGG'.

Tips:

- Choose spacers with cut site (3 bp 5' of 'NGG') as close to the intended genetic modification as possible.
- Design at least 2 different spacers, as sgRNA efficiency can vary.

This information is not exhaustive. Useful guides on gRNA design and CRISPR-Cas9 use:

- [How to Design Your gRNA for CRISPR Genome Editing - Addgene](#)
- [CRISPR Guide RNA Design Guidelines for Efficient Genome Editing](#)
- [CRISPR guide RNA design for research applications](#)

- 2 Order two 60 bp complementary oligos that will be annealed:
 - 60 bp forward oligo with the desired sgRNA spacer sequence (ctagacacctgcagcgggacNNNNNNNNNNNNNNNNNNNNgtttcgaggcaggtgcttcc, where "NNN...NNN" is the spacer sequence)
 - 60 bp reverse oligo with desired sgRNA spacer reverse complement sequence (ggaagcacctgcctcgaaacNNNNNNNNNNNNNNNNNNNN gtcccgctgcaggtgtctag, where ("NNN...NNN" is the reverse complement of the spacer sequence)

GeneBank files of annealed oligos:  empty_spacer_annealed_oligos.gbk 1.7KB

- 2.1 We have also made an excel spreadsheet that automatically generates spacer oligos in bulk, starting from spacer sequences:  sgRNA_spacer_oligogenerator.xlsm 26.6KB

Design homology-repair template

- 3 Homology-repair template design: 
 - For nucleotide-level editing, we recommend to use a 60 bp double-stranded DNA template. Design two complementary oligonucleotides ("oligos") that include the modified bases around the middle.
 - For tagging: Design two primers that amplify the tagging cassette of choice and have an overhang of 30 bp that acts as homology arm to the insertion locus.



Necessary criteria:

- The final genetic modification in the repair template should include changes to either the PAM or the protospacer to prevent Cas9 from recognising the site and cutting it again. Mutations of either G base within the PAM are usually preferable.

3.1 Order the designed oligos.

Oligo Annealing

- 4 If ordered dry, resuspend oligos to 100 μ M in water or TE buffer.
- 5 Mix 2.5 μ L of forward oligo, 2.5 μ L of reverse oligo and 5 μ L of annealing buffer (100 mM NaCl, 20 mM Tris pH 7.5) in a 0.2mL PCR tube. Final oligo concentration will be 25 μ M.
- 6 Place the tube in a thermocycler and run the following program:
 1. 3 minutes at 95°C.
 2. Ramp-down 1°C every 30 seconds until reaching 25°C (70 cycles).
 3. Hold at 10°C.
- 7 Mix 0.5 μ L of annealed oligos with 875 μ L of water (1:1750 dilution) in a 1.5 mL microcentrifuge tube.

PaqCI Golden Gate Reaction

- 8 Prepare the following reaction in a 0.2mL PCR tube on ice:

Reagent	Volume
Annealed spacer oligonucleotides (~1 ng/ μ L)	0.5 μ L
CRISPR-Cas9 vector plasmid (75 ng/ μ L)	0.5 μ L
PaqCI	0.25 μ L
PaqCI activator	0.125 μ L
T4 Ligase	0.25 μ L
T4 Ligase buffer	1 μ L
Water	7.375 μ L



- 9 Run the following program on the thermocycler:
 1. 60 minutes at 37°C.
 2. 5 minutes at 60°C.

Note

The reaction can be stored at -20°C. If storing the reaction at -20°C, the 5 mins 60°C incubation should be repeated after thawing.

Plasmid transformation and verification

- 10 Transform 2.5 µL into DH5α *E. coli* according to your preferred method.

- 11 Plate on LB + Ampicillin (100 µg/mL) agar plates.

Note

The transformation is usually very efficient. If doing a typical bacterial transformation, after heat-shock and 45-minute recovery at 37°C, we usually plate 10% of the transformation reaction.

Alternatively, the recovery step of the transformation can be skipped and the whole transformation plated. This will produce more background empty CRISPR-Cas9 vector plasmid colonies, but will still give enough sgRNA plasmid colonies.

- 12 Incubate plates at 37°C overnight.



- 13 Start a culture from a non-fluorescent bacterial colony in liquid LB + Amp (100 µg/mL).

Note

If the sgRNA Golden Gate cloning has worked correctly, the GFP cassette will have been removed from the plasmid, and the bacteria will not express GFP.

It is usually easy to tell which colonies produce GFP as they look light green in ambient light.

Alternatively, plates can be exposed to blue light (e.g. from Blue-light gel transilluminator) which will make the difference between fluorescent and non-fluorescent colonies more obvious.



14 Incubate at 37°C overnight, shaking at 180 rpm.



15 Isolate the plasmid according to your preferred method. Ideally, plasmid concentration should be >800 ng/μL for efficient *A. nidulans* transformation.

Plasmid verification

16 Correct insertion of the protospacer can be verified by Sanger sequencing using primer oDM154: TTGAGCAAACCTCTGATCGCCTG.

Note

There is very little chance of errors in the Golden Gate reaction, and we usually proceed with *A. nidulans* transformation before we get Sanger sequencing results for the plasmid.

Repair template preparation

17 Prepare the repair template for your intended genomic modification.

For nucleotide-level editing: order two complementary oligos and anneal as in step 5 and 6. The reaction can be transformed as is.

For tagging: amplify the tagging cassette by PCR with primers with a 30 bp overhang which code for the homology arms. The reaction should be cleaned up before transformation.

Checking sgRNA efficiency

18 We recommend testing the sgRNA efficiency by Technique to Assess Protospacer Efficiency (TAPE) as described by Nødvig et al, 2018:



1. Transform 1 - 2 μg of your CRISPR-Cas9 sgRNA plasmid without a repair template into NHEJ-deficient (Δ kuA) *A. nidulans*
2. The efficiency of the sgRNA will be inversely proportional to the number of colonies on the transformation plate, as the organism will not be able to repair a double-stranded break without a repair template.
3. We recommend using sgRNA plasmids that produce few to no colonies via TAPE.



Note

TAPE can be done in parallel to the transformation including the repair template. We usually test two sgRNAs both with and without the homology-repair template. We proceed with strain verification only if the plates with no repair template produce few to no colonies.

Citation

Nødvig CS, Hoof JB, Kogle ME, Jarczynska ZD, Lehmbeck J, Klitgaard DK, Mortensen UH (2018). Efficient oligo nucleotide mediated CRISPR-Cas9 gene editing in *Aspergilli*.

<https://doi.org/10.1016/j.fgb.2018.01.004>

LINK

A. *nidulans* transformation

- 19 Transform your plasmid and repair template into *A. nidulans* according to your preferred transformation method. In the steps below, we reproduce the protocol described in Oakley et al., 2012 with minor modifications.

We usually transform 1 - 2 µg plasmid and 0.6 - 1.5 µg of purified homology-repair template in a volume of 6 µL per 40 µL of protoplasts.

Citation

Oakley CE, Edgerton-Morgan H, Oakley BR (2012)
. Tools for manipulation of secondary metabolism pathways: rapid promoter replacements and gene deletions in *Aspergillus nidulans*.

https://doi.org/10.1007/978-1-62703-122-6_10

LINK

- 20 Prepare the following solutions:

1.2 M Sucrose:

- Prepare 250 mL of 1.2 M sucrose.
- Autoclave and store at 4°C.

KCl citrate solution:

- Dissolve 8.2 g of KCl and 2.1 g of citric acid monohydrate in approximately 50 mL dH₂O.
- Adjust to pH 5.8 with 1.2 M KOH (made fresh before use).
- Transfer to a clean graduated cylinder and add dH₂O to final volume 100 mL.
- Autoclave and store at room temperature.

0.6M KCl solution:

- Prepare 100 mL of 0.6 M KCl.
- Autoclave and store at room temperature or 4°C.

0.6M KCl 50 mM CaCl₂ solution:

- Prepare 100 mL of 0.6 M KCl, 50 mM CaCl₂.
- Autoclave and store at room temperature or 4°C.

PEG solution (0.6M KCl, 50 mM CaCl₂, 25% PEG 3,350, pH ~7.5) :

- Dissolve 4.47 g of KCl, 0.74 g of CaCl₂ dihydrate in 20 mL of dH₂O in a 100 mL graduated cylinder.
- Add 0.804 mL 1.0 M Tris-HCl, 0.196 mL 1.0 M Tris base and mix well.
- Add dH₂O to 90 mL. Seal top with parafilm and mix by inversion at 10-min interval until PEG is fully dissolved.
- Add dH₂O to 100 mL, seal and mix by inversion.
- Syringe-filter solution with a 0.22 µm PVDF syringe filter (multiple filters will be necessary), aliquotting into 15 mL conical tubes. The solution should be re-filtered on the day of transformation.
- Store at room temperature.

- 21 Inoculate 40 mL of nonselective media in a 100 mL Erlenmeyer flask with $\sim 2 \times 10^8$ conidia. We usually use rich medium (YG + supplements if necessary).

Note

Protoplast yield seems to be higher when the conidia are harvested on the same day it is used for inoculation.



- 22 Incubate for ~16 h at 30°C shaking at 120 rpm.
- 23 Before harvesting the culture, prepare the 2x protoplasting solution. Dissolve 4 g of Laffort Extralyse in 20 mL of 1.2 M KCl/citrate solution in a 50 mL centrifuge tube. The enzyme mix will dissolve in around 15-20 minutes on a roller, with occasional vortexing.
- 24 Centrifuge the protoplasting solution at 5,000 g (or centrifuge max speed if below 5,000 g) for 15 min at room temperature.
- 25 Remove the lipid layer from the top of the solution by pipetting it out with a 1000 µL tip.
- 25.1 Syringe-filter the solution with a 0.22 µm low protein binding filter unit.
- 26 Harvest the hyphae by filtering the culture through Miracloth (cut into 5 cm squares and autoclaved within aluminum foil). Wash with at least 3× the culture volume of sterile deionized water. Gently squish the mycelium with a flamed spatula to get rid of excess water.
- 27 Transfer mycelium into a 50 mL flask with 16 mL non-selective medium and 16 mL 2x protoplasting solution. Resuspend thoroughly by pipetting up and down with a 1 mL sterile plastic pasteur pipette until most mycelium clumps have disassembled.
- 28 Incubate at 30°C, shaking 100 rpm for 1.5-2 h.
- 28.1 Resuspend clumped mycelium as above every 15 minutes. Formation of protoplasts can be checked by phase contrast microscopy. During this step start pre-cooling a centrifuge to 4°C.
- 29 Place flask on ice and shake at 100 rpm for 5 minutes. This can be done by putting an ice bucket on a benchtop shaker.
- 30 Pipette 15 mL of cold 1.2 M sucrose into two 50 mL centrifuge tubes (15 mL per tube).
- 31 Gently pipette ~10 mL of protoplast on top of the sucrose solution, on ice.

Note

For best results keep 50mL tubes at angle on ice and pipette down the protoplast using a 10

mL pipette. Pipette down against the wall of the tube.

If you have use a pipette controller with a force-sensitive button, gentle pipetting can be achieved by very gently pressing the pipette up button (not pressing hard enough to turn on the

motor). This will lead the liquid to fall by gravity.

Alternatively, we also achieved good layering by using a 25mL Eppendorf combitip with pipette-

down speed setting set to 1.

Always handle the sucrose cushion tubes with care.

- 32 Set the cooled centrifuge for slow acceleration and deceleration. We set both at 3 in a scale 0-9 (where 9 is fastest acceleration and deceleration) in both Eppendorf and Thermo Fischer centrifuges.

- 32.1 Centrifuge at 2,800 g for 10 mins at 4°C.

- 33 Protoplast will accumulate at the interface of the two phases, forming a thin cloudy layer.

Harvest ~3mL of protoplast from the interface using a 1000 µL pipette and collect in a 15 mL

conical tube. Use Eppendorf Protein Lo-Bind tubes to reduce protoplast loss.

Note

You can harvest from the interface even if a cloudy layer is not visible, but protoplast yield will likely be lower.

- 33.1 Add cold 0.6 M KCl to 15 mL.

- 34 Centrifuge at 2,300 for 10 mins at 4°C.



- 35 Gently pour away supernatant and discard. Resuspend protoplast pellet in 2 mL of cold 0.6 M KCl. Divide into two 1 mL aliquots in 1.5 mL Eppendorf protein Lo-Bind microcentrifuge tubes.
- 35.1 Centrifuge at 2,400 g for 3 mins at 4°C in a pre-cooled microcentrifuge.
- 36 Gently pipette out supernatant and resuspend in 1 mL cold 0.6 M KCl.
- 37 Centrifuge at 2,400 g for 3 mins at 4°C.
- 38 Discard supernatant, resuspend in 500 µL cold 0.6 M KCl 50 mM CaCl₂ and combine the two aliquots in the same tube.
- 39 Centrifuge at 2,400 g for 3 mins at 4°C.
- 40 Gently pipette out supernatant, and resuspend pellet in 40 µL per transformation to be done (e.g. for five transformations, resuspend in 200 µL plus 40 µL extra).
- 41 If the protoplast yield is high, extra protoplasts can be frozen by adding DMSO to final concentration of 7% (v/v) and storing at -70°C without any special treatment.

*

When using frozen protoplasts, thaw on ice and repeat a wash in 0.6 M KCl 50 mM CaCl₂.

Frozen protoplasts have a reduced transformation efficiency. As a rule of thumb, if freezing 400 µL (usually for 10 transformations), resuspend in half the volume (200 µL) after thawing and use for up to 5 transformations.
- 42 Aliquot 40 µL protoplast into 1.5 mL microcentrifuge tubes, one tube per transformation.
- 43 In each tube add 1 - 2 µg of CRISPR-Cas9 sgRNA plasmid and 0.6 - 1.5 µg of purified homology-repair template, up to a combined volume of 6 µL to maintain osmotic balance.
- 43.1 Mix by vortexing in 6 one-second bursts.



- 44 Add 20 μL of room-temperature PEG solution. On the day of transformation, the PEG solution should be syringe-filtered with a 0.2 μm PVDF unit.
- 44.1 Mix by vortexing in 6 one-second bursts.
- 45 Place on ice-water bath for 25 mins.
- 46 Add 400 μL of PEG solution (filtered on the day of transformation).
- 46.1 Mix by slowly pipetting up and down 10 times.
- 47 Incubate at room temperature for 30 mins.
- 48 Spread onto appropriate selection plates that also contain 1 M sucrose. Use 150 μL per plate, and spread using glass beads.
- 49 Incubate at 30°C for 1 day.
- 50 Incubate at 37°C for 2-3 days.



Spore dissection of transformants

- 51 We purify the strain by spore dissection on a non-selective agar plate using a Singer MSM 400 Tetrad Dissection Microscope (Singer, USA); other yeast dissecting scopes should also be suitable.

Note

Alternatively, strains can be purified by three rounds of streak-purification.

- 51.1 Pick spores from transformants. There are two different ways to do this:



- If the colonies have visible conidia, we pick some conidia with a toothpick and resuspend them in 30 μL of water in a 0.2 mL tube.
- If the colonies do not have visible conidia, we pipette 5 μL of water up and down the center of a colony a few times.

51.2 Draw a straight line on the bottom part of the non-selective plate and drop 5 μL of spore suspension below it. Then make a streak from the drop with a toothpick and let dry.

Multiple drops (usually 3-4) can fit in a single plate.

51.3 Mount the plate onto the microscope and dissect around 3-4 spores from each dried spore suspension patch.

51.4 Once single spores are dissected, cut the bottom part of the agar along the line and discard it.

52 Incubate at 37°C. Colonies usually form after 2-3 days.

Strain verification

15m

53 Verify the strain by genotyping PCR and sequencing.

In the steps below, we perform spore PCR from spores as described by Fraczek et al., 2019.

We verify gene insertion by amplifying the genomic region of interest using forward and reverse primers that anneal 5' and 3' of the insertion site, respectively. Initially, we perform PCR using a Taq-based polymerase and visualize the product using DNA gel electrophoresis. Successful insertion should produce a higher molecular weight band compared to the wild-type strain. For candidate-positive strains, we repeat the PCR using a high-fidelity polymerase and sequence the product via Sanger or nanopore amplicon sequencing.

To verify precise editing, we simply amplify the edited genomic region and send it directly for sequencing. This process allows us to confirm both the presence and accuracy of gene insertions or edits.

Note

We find that primers picked using **Primer-BLAST** work very well.



Citation

Fraczek MG, Zhao C, Dineen L, Lebedinec R, Bowyer P, Bromley M, Delneri D (2019) . Fast and Reliable PCR Amplification from *Aspergillus fumigatus* Spore Suspension Without Traditional DNA Extraction.

<https://doi.org/10.1002/cpmc.89>

LINK

- 53.1 Pick spores from the single colonies with a 200 μ L pipette tip. A ~0.5 cm streak over the conidiated part of the colony is usually enough.

Note

Keeping the tip at an angle makes the conidia accumulate to its side rather than inside it; this will make resuspension in water easier later.

- 53.2 Resuspend the spores in 30 μ L of water in a 0.2 mL tube.

Expected result

The spore suspension colour is slightly cloudy to light green.

- 53.3 In a thermocycler, boil the spores at 95°C for 15 minutes.

- 53.4 Quickly move the spores to -70°C and leave for at least 15 minutes.



Note

After this step, the spore suspension can be stored at -70°C or -20°C and used as a PCR template multiple times.



53.5 Perform the PCR using 3 μ L of boiled-frozen suspension as template.

For genotyping via DNA gel electrophoresis we use

 LongAmp Taq DNA Polymerase - 500 units **New England Biolabs Catalog #M0323S**

as described in Fraczek et al., 2019.

For high-fidelity PCR we use

 Platinum™ SuperFi II DNA Polymerase **Invitrogen - Thermo Fisher Catalog #12361010**

according to the manufacturer's protocol.

53.6 Send high-fidelity PCR product for sequencing as required.

54 Make a long-term stock of your final strain according to your preferred method. We store *A. nidulans* spore suspension at -70°C with glycerol at a final concentration of 16% (v/v).

Protocol references

Oakley, C. E., Edgerton-Morgan, H. & Oakley, B. R. Tools for Manipulation of Secondary Metabolism Pathways: Rapid Promoter Replacements and Gene Deletions in *Aspergillus nidulans*. in *Fungal Secondary Metabolism: Methods and Protocols* (eds. Keller, N. P. & Turner, G.) 143–161 (Humana Press, Totowa, NJ, 2012). doi:[10.1007/978-1-62703-122-6_10](https://doi.org/10.1007/978-1-62703-122-6_10).

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Citations

Step 18

Nødvig CS, Hoof JB, Kogle ME, Jarczyska ZD, Lehmbeck J, Klitgaard DK, Mortensen UH. Efficient oligo nucleotide mediated CRISPR-Cas9 gene editing in *Aspergilli*.

<https://doi.org/10.1016/j.fgb.2018.01.004>

Step 19

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https://doi.org/10.1007/978-1-62703-122-6_10

Step 53

Fraczek MG, Zhao C, Dineen L, Lebedinec R, Bowyer P, Bromley M, Delneri D. Fast and Reliable PCR Amplification from *Aspergillus fumigatus* Spore Suspension Without Traditional DNA Extraction.

<https://doi.org/10.1002/cpmc.89>