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Transformation Aspergillus niger using Cas9, AMA1 vector, pyrG rescue marker and sgRNA in vitro.

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We use this protocol and it's working

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Troubleshooting

Ahead before the transformation

1 -Prepare the construct containing the marker pyrG. We used the Phusion Hot Start II to amplify the plasmid containing the construct. The PCR product is purify and concentrate to 1ug/uL and we need 10ug for the transformation.

-Prepare the plasmid containing the Cas9 cassette, the hygromycin marker and the AMA1 cassette (replication). Concentrate the plasmid to 1ug/uL and we need 1ug for the transformation.

-Verify the sgRNAs in vitro before the transformation. To do so, they were prepared and tested in vitro using the Guide-it sgRNA Screening Kit (Takara), After the sgRNA are validating (follow Takara protocol), they are amplify by PCR using the GeneART gRNA synthesis (Follow GeneART gRNA synthesis protocol).

-Per transformation plan Minimal Media Agar (MMA) Plate (10 g/L Glucose, 50 ml/L 20X Nitrate Salts Stock Solution, 1 ml/L 1000X Trace Elements Stock Solution, 1 ml/L 1000X Vitamins Stock Solution, pH 6.5 using KOH, 18 g/L Granulated Agar, 1M of Sorbitol) with hygromycin 300 ug/mL. Plan also for positive control and negative control plate.
Also per transformation plan MMA containing hygromycin in solution placed at 60^{o}C to maintain liquid, plan 10mL per transformations (controls included).

The day before the transformation

2 -In order to begin generating protoplasts for transformation, spored were harvestd from one MMA Agar slants tubes of the A. niger strain using 0.2% Tween-20 solution and counted with hemocytometer

-The strain (pyrG $^{-}$)was then inoculated at a concentration of 5×10^{5} spores/mL into 100mL of YPD with urcail 1.2 mg/mL growth medium in a 250-mL glass flask

-The flask was then capped and incubated overnight in a shaker set at 150 rpm at 30^{o}C

The day of the transformation

3 Preparation of the protoplast:

-Freshly prepare 40 mL of 30 mg/mL VinoTaste Pro (from Novozymes) in Protoplasting Buffer (0.6 M ammonium sulfate and 50 mM maleic acid in ddH2O, pH adjusted to 5.5) (note: this can take a little while to go into solution so leave on stir-plate while harvesting the mycelia)

-Filter-sterilize the VinoTaste Pro/Protoplasting Buffer solution using a 0.45 µm bottle-top vacuum filter

-The overnight YPD culture of the A. niger strain was filtered through a single layer of sterilized Miracloth (from Millipore) using a Buchner funnel and gravity filtration system (into a sterile 500-mL glass bottle)

-The mycelia retained on the Miracloth was rinsed with three times with 100 mL sterile ddH2O; the flow-through was discarded

-The washed mycelia was then transferred to a sterile 250-mL glass flask containing 40 mL of the filter-sterilized solution of the VinoTaste Pro/Protoplasting Buffer solution

-The digesting mycelia were incubated in a shaker set at 70 rpm at 30 °C for 4-6 hr; every 1-2 hr a small aliquot of the solutions was examined using a light microscope to detect the progress of protoplast formation

4 Preparation of the sgRNA *in vitro*:

-While the mycelia are getting digested, there is enough time to prepare the sgRNA *in vitro*.

-To prepare the sgRNA *in vitro* from the template, use the GeneART protocole to transcript and purify. The concentration obtained is between 5 ug/uL and 10 ug/uL, we use 20 ug per transformation (if one sgRNA is used, use 20ug, if two sgRNAs are used, use 10ug/sgRNA). Save of ice for the transformation.

5 Purifying the protoplasts for the transformation:

-When a significant quantity of protoplasts had been generated (~5 hr post-inoculation), the digested mycelia solution was decanted over a single layer of sterilized Miracloth in a 50-mL conical tube; the mycelia retained on the Miracloth was discarded

-The flow-through was centrifuged at 800 rcf for 10 min and the resulting supernatant decanted and discarded

-In order to wash the protoplasts, the pellet was first resuspended in 1 mL ST Solution (1 M sorbitol in 50 mM Tris, pH 8.0) by pipetting up and down using a wide-bore pipette tip;

25 mL ST Solution was then added to the sample, which was again centrifuged at 800 rcf for 10 min and the resulting supernatant decanted and discarded

-The protoplast pellet was again resuspended in first 1 mL and eventually 25 mL ST Solution before being centrifuged at 800 rcf for 10 min and the resulting supernatant decanted and discarded

-The protoplast pellet was next resuspended in 200 uL STC Solution (1 M sorbitol and 50 mM CaCl2 in 50 mM Tris, pH 8.0) by pipetting up and down using a wide-bore pipette tip. Save on ice for the transformation (this should be enough for 3 to 4 transformations, controls included).

6 THE TRANSFORMATION:

-In a falcon tube (12mL) , add the protoplast (*A. niger pyrG* $^{-}$)100uL
-Add into the protoplast: 1ug of Cas9 plasmid (hph marker), 20ug of sgRNA(s), and the 10ug of construct (pyrG) and 100 uL of transformation solution (25% PEG 6000, 50 mM CaCl2 and 10 mM Tris-HCl, pH 7.5) and incubate on ice for 20 min.
(Note: for the positive control, we used two plasmids containing each the markers, pyrG and hph. For the negative control, we used just the plasmid hph and not the pyrG construct)

-Add 2 mL of transformation solution and incubate 5 min at room temperature (RM). During this time, put out the MMA + hph out of the oven to let it cool gently down to ~45C.

-Add 4 mL STC and mix by inverting the tube several times gently.

-Add 7mL of the molten top agar to transformation mixtures, mix by pipetting up and down and immediately pour onto selection plate.

Uncubate at 30^{O}C for 4 days until the sporulation.

After the transformation

7 -After visible growth but before the appearance of the first spores, the colonies were scooped out and isolated on slants containing only MMA.The Cas9 plasmid is lost in the absence of selective pressure (hygromycin).

-Once the colonies in the slants formed spores, the spores were isolated on plates containing MMA + 1.3 mg/mL 5-FOA + 1.2 mg/mL uracil. (To isolate colonies, we harvest spores into 0.2% tween-20 solution 100 uL, and dilute 1:10 into 100 uL solution, 10 uL of the 1:10 solution is plated on 5-FOA).

-If the colonies grow (meaning the pyrG is well inserted), they are re-isolated using MMA + 1.3 mg/mL 5-FOA + 1.2 mg/mL uracil plates again, then before the appearance of the first spores the colonies are scooped out and place on slants containing MMA + 1.2 mg/mL uracil/uridine.

-For each transformation a minimum of 10 colonies are isolated, transformed on 5-FOA then re-isolated for analysis by PCR and sequencing.
Note that if the pyrG marker needs to be recycled, it is recommended that the fungi recover between experiments. Also, manipulation of spores often leads to contamination and requires great care during the transformation.