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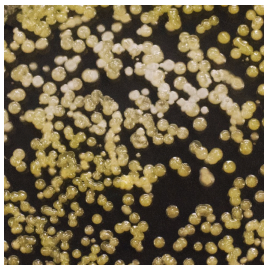
CRISPR/Cas9 based knockout generation in Aurantiochytrium limacinum (ATCC MYA-1381)



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Protocol status: Working
We use this protocol and it's working

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Abstract

Transformation protocol for electroporation of CRISPR/Cas9 ribonucleoprotein (RNP) complex in urantiochytrium limacinum (ATCC MYA-1381; Stramenopile/ Heterokont, Thraustochytrid).

Materials

MATERIALS

- NEPA21 Super Electroporator **NEPAGENE**
- EnGen® sgRNA Synthesis Kit S. pyogenes **New England Biolabs** **Catalog #**E3322S
- Cas9 Nuclease S. pyogenes **New England Biolabs** **Catalog #**M0386S
- Monarch® RNA Cleanup Kit **New England Biolabs** **Catalog #**T2050S

STEP MATERIALS

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Designing oligos for gRNA synthesis

40m

1 Use ChopChop tool (chopchop.rc.fas.harvard.edu) to select the gRNA of interest. *Aurantiochytrium limacinum* genome is available on ChopChop, and the gene of interest can be accessed using the gene co-ordinates of the JGI assembly https://mycocosm.jgi.doe.gov/Aurli1/Aurli1.home.html(For example: **scaffold_10:1222284-1223740 (+)**).

10m

2 The output will list the gRNAs in a ranked order, considering the self-complementarity of the gRNA, efficiency, and off-target effects. Select the highly ranked gRNA and proceed for oligo designing for in vitro transcription.

10m

3

10m

Note

Design target specific oligos following the **NEB protocol**

Select 20 nt gRNA sequences using the ChopChop web tool (do not include the PAM sequences), and add 'G' to the 5' end of the sequence, only if there is no 'G' at the 5' end.

4 To the 5´ end; append T7 promoter sequence: **TTCTAATACGACTCACTATA**
To the 3´ end; append 14 nucleotide overlap sequence: **GTTTTAGAGCTAGA**
The final oligo sequence should look like 5´ **TTCTAATACGACTCACTATAG**$(N)_{20}$**GTTTTAGAGCTAGA** 3´, $(N)_{20}$ is the gRNA sequence.
This is the sequence of the oligo to be ordered.

10m

Note

IMPORTANT: While the oligos are shipped, we recommend starting the pre-culture (step8) at this stage since it takes 2-3 days to grow.

gRNA synthesis and purification

1h 30m

5

1h

Note

Wear gloves and use nuclease-free tubes and reagents. Reactions should be assembled in microfuge tubes or PCR strip tubes.

Perform gRNA synthesis following the **EnGen®sgRNA Synthesis Kit**.

EnGen® sgRNA Synthesis Kit S. pyogenes **New England Biolabs Catalog #**E3322S

6 Purify the synthesized gRNA using Monarch RNA Cleanup Kit (50 µg) 30m

Monarch® RNA Cleanup Kit **New England Biolabs Catalog #**T2050S

Quantify the gRNA using UV-Vis Nano-spectrometer.
Label and store the gRNA at -20 °C for short term storage and at -80 °C for long term storage.

Ribonucleoprotein (RNP) preparation 30m

7 Thaw the gRNA and 1x PBS on ice. Spin down all the reagents before using. 30m

For a total reaction volume of 5µl,
1. Add [M] 120 pmol (final) gRNA and [M] 104 pmol (final) Cas9 protein.
2. Adjust the reaction volume to 5µl using 1x PBS.

Cas9 Nuclease S. pyogenes **New England Biolabs Catalog #**M0386S

Mix the reagents by gently pipetting up and down. After a brief spin, incubate the tube at Room temperature for 00:20:00 .

Label and store the RNP (Cas9/gRNA) complex in -20 °C until further use.

Note

Optional
Check the efficiency of RNP by performing an in vitro cleavage assay.

Grow cells

4d

8

4d

1. Start a pre-culture 96:00:00 (4 days) prior to electroporation by inoculating 5 mL of GPY (0.5% Yeast Extract, 1% Peptone, 3% D+-Glucose, 1.8% instant ocean) in 15 mL tube with a colony of *Aurantiochytrium limacinum* (ATCC MYA-1381). Incubate Overnight at 28 °C .
2. Use preculture to inoculate 46 mL of GPY in 250 mL flask. Culture for 3 days at 28 °C , 171 rpm .

Prepare reagents for Electroporation

2h

9

2h

1. Prepare GPYs media (3% glucose, 0.6% peptone, 0.2% yeast extract, [M] 50 millimolar (mM) sucrose, 1.8% instant ocean)
2. Prepare GPYs ampiciln (100µg/ml) plates (2%agar)
3. Prepare GPYs zeocin (100µg/ml) and ampiciln (100µg/ml) plates (2%agar)

Note

GPYs zeocin + ampicillin plates are used if homology directed repair (HDR) template with Zeo^R is used.

1. Prepare 1X BSS ([M] 10 millimolar (mM) KCl, [M] 10 millimolar (mM) NaCl, and [M] 3 millimolar (mM) CaCl2)
2. Prepare [M] 50 millimolar (mM) sucrose solution
3. Sterilize by autoclave or filter sterilization as appropriate.

CITATION

Mariana Rius, Jackie Collier. Electroporation of Aurantiochytrium limacinum (ATCC MYA-1381).

LINK

dx.doi.org/10.17504/protocols.io.qjcduiw

Prepare cells for electroporation

45m

10

45m

1. Count cells using haemocytometer. Cell density should be around ~ 5 ×10^7 cells/ml.
2. Add 1.5 ml of cells to a microcentrifuge tube for each electroporation reaction. Centrifuge
3. 11000 rpm, 4°C, 00:05:00
4. Decant the supernatant. Add 500 ul chilled 1X BSS ([M] 10 millimolar (mM) KCl, [M] 10 millimolar (mM) NaCl, and [M] 3 millimolar (mM) CaCl2). Centrifuge 11000 rpm, 4°C, 00:05:00
5. Decant the supernatant. Add 500 µL chilled [M] 50 millimolar (mM) sucrose. Centrifuge 11000 rpm, 4°C, 00:05:00 . Repeat thrice (3x).
6. Re-suspend by scraping cell mass off side of tube and vortex.

Add RNP

5m

11

5m

1. Add 5 µL of RNP complex mixture to suspended cells.
2. Incubate on ice 00:05:00 .
3. Transfer to chilled electroporation cuvette (0.2cm; BioRad).
4. Keep the cuvettes on ice.
5. Keep appropriate negative control using the same volume of elution buffer or water.

Electroporation

10m

12

10m

Equipment

NEPA21 Super Electroporator	NAME
NEPAGENE	BRAND
NEPA21	SKU
http://www.nepagene.jp/e_products_nepagene_0001.html	LINK

Clean the loading pedestal and the cuvette before loading.

Set the parameters for poring and transfer pulse,
Poring pulse:

	Voltage (V)	Pulse length (ms)	Length interval (ms)	No. of pulse	Decay rate (%)	Polarity
	275	8	50	2	10	+

Transfer pulse:

	Voltage (V)	Pulse length (ms)	Length interval (ms)	No. of pulse	Decay rate (%)	Polarity
	20	50	50	1	40	+/-

The capacitance and resistance is set at 125 µF, and 1000 Ω respectively.

Pulse the cells and record the time constant. Take out the cuvette and place it on ice.
Repeat the steps for the rest of the samples.

Outgrowth and plating

1h 30m

13 1h 30m

1. Add 1ml GPYs media to the cuvette and carefully aspirate out the cells on to a fresh micro centrifuge tube.
2. Label the corresponding tubes and Incubate at 28 °C for 01:00:00 without shaking.
3. Centrifuge the cells at 11000 rpm, 25°C, 00:05:00 , and discard the supernatant.
4. Re-suspend the cells in remaining supernatant by pipetting up and down and also by vortexing.
5. Dilute the cells appropriately (100x, 1000x) in 100 µL of fresh GPYs media.
6. Plate the cells on appropriate GPYs plates, we usually include ampicillin to control bacterial contamination, and incubate at 28 °C .

Monitor transformant colonies

2d

14 2d

Colonies of *Aurantiochytrium limacinum* transformants will appear 2 days after plating. Streak individual colonies on fresh GPYs plate, and observe the phenotype/genotype. Include wild type (WT) for comparison.

Note

The transformant selection/screening can vary depending on the target gene and the selection marker used. In our case, we knocked-out carotenogenic gene, and the phenotype selection was based on colony colour.

Citations

Step 9

Mariana Rius, Jackie Collier. Electroporation of Aurantiochytrium limacinum (ATCC MYA-1381) **dx.doi.org/10.17504/protocols.io.qjcduiw**