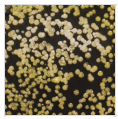

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CRISPR/Cas9 based knockout generation in Aurantiochytrium limacinum (ATCC MYA-1381)

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1 Works for me dx.doi.org/10.17504/protocols.io.baeyibfw

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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

NAME	CATALOG #	VENDOR
NEPA21 Super Electroporator	View	NEPAGENE
EnGen® sgRNA Synthesis Kit S. pyogenes	E3322S	New England Biolabs
Cas9 Nuclease S. pyogenes	M0386S	New England Biolabs
Monarch® RNA Cleanup Kit	T2050S	New England Biolabs

STEPS MATERIALS

NAME	CATALOG #	VENDOR
EnGen® sgRNA Synthesis Kit S. pyogenes	E3322S	New England Biolabs
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Designing oligos for gRNA synthesis 40m

1 10m

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 (+)).

2 10m

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.

3 10m

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** 10m
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.

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

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
by New England Biolabs
Catalog #: E3322S

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

Monarch® RNA Cleanup Kit
by 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 ⚠ 30m

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

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
by 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.

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

Grow cells 96h

8 96h

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 Milimolar (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)

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

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

Mariana Rius, Jackie Collier. Electroporation of Aurantiochytrium limacinum (ATCC MYA-1381).
http://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 x10^7 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 Milimolar (mM) KCl, [M]10 Milimolar (mM) NaCl, and [M]3 Milimolar (mM) CaCl2). Centrifuge 11000 rpm, 4°C 00:05:00
5. Decant the supernatant. Add 500 µl chilled [M]50 Milimolar (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

NEPA21 Super Electroporator

NEPAGENE NEPA21

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 48h

14 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. 48h

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.