Jun 21, 2021

Microparticle delivery of CRISPR-Cas9 ribonucleoprotein to *Isochrysis galbana* for gene editing of *URA3* gene.

Andrea Highfield[1], Glen Wheeler[2]

[1]The Marine Biological Association of the United Kingdom; [2]Marine Biological Association

In Development Share dx.doi.org/10.17504/protocols.io.bvxen7je

Protist Research to Optimize Tools in Genetics (PROT-G)

Glen Wheeler

ABSTRACT

This is a method for gene editing in Isochrysis galbana. It uses a ribonucleoprotein (RNP) approach to introduce Cas9 into I. galbana for targeted gene editing, with selection for resistance to 5-FOA. The method is promising as we have good evidence that editing is occurring in the 5-FOA resistant colonies, although we have not yet been able to recover stable individual clones. The method therefore requires further optimisation before it can be used routinely.

DOI

dx.doi.org/10.17504/protocols.io.bvxen7je

PROTOCOL CITATION

Andrea Highfield, Glen Wheeler 2021. Microparticle delivery of CRISPR-Cas9 ribonucleoprotein to Isochrysis galbana for gene editing of URA3 gene.. **protocols.io** https://dx.doi.org/10.17504/protocols.io.bvxen7je

CREATED

Jun 17, 2021

LAST MODIFIED

Jun 21, 2021

PROTOCOL INTEGER ID

50886

Prepare Isochryis galbana CCMP 1323 cells

1 Prepare 50% salinity F/2; 1 % (w/v) agar plates in advance. These can be stored for up to a month at 4oC. Ensure plates are dried in a sterile hood before use.

2 Grow *Isochrysis galbana* cells in 50% salinity F/2 liquid media under constant illumination at 18°C.

3 Harvest cells when they reach log phase, at a density of approximately 1 x 10^6 cells mL^{-1}.

4 On the day that you plan to undertake microparticle bombardment, centrifuge cells in 50 mL falcon tubes at 18°C for 15 minutes at 3000 rpm.

5 Discard supernatant and re-suspend pellet in 250 µL 50% salinity F/2.

6 Plate cells on a 50% salinity F/2 agar plate by pipetting cells into the centre and gently rotating the plate so the cells cover approximately 3 cm in diameter.

7 Allow to dry.

Prepare tungsten microparticles

8 Weigh out 60mg tungsten in a microfuge tube. This stage can be done in advance.

9 Wash in 1mL 100% molecular grade ethanol by vortexing (1-5min) then centrifuge at 13000 rpm for 1 minute.

10 Remove supernatant and add 1 mL sterile molecular grade water. Vortex, centrifuge at 13000 rpm for 1 minute and remove supernatant.

11 Repeat step ten a further two times.

12 Re-suspend beads in 1 mL sterile molecular grade water and aliquot into 50 µL volumes whilst vortexing continuously to avoid sedimentation of beads.

13 Store aliquots at -20°C.

Prepare RNP complex

14 Make 100 µM stocks of tracrRNA and crRNA. Mix 1µL tracrRNA with 1µL crRNA.

15 To form RNP complex prepare the following (final volume 8µL):
4 µL crRNA/tracrRNA complex
25 µL Cas9 (4µg)
8 µL NEB buffer 3.1
55 µL water

Citation: Andrea Highfield, Glen Wheeler (06/21/2021). Microparticle delivery of CRISPR-Cas9 ribonucleoprotein to Isochrysis galbana for gene editing of URA3 gene.. https://dx.doi.org/10.17504/protocols.io.bvxen7je

16 Incubate at room temperature for 20 minutes. Can then be stored at 4°C.

17 Wash tungsten beads in 1 mL 1 x NEB buffer 3.1. Centrifuge at 13000 rpm for 1 minute and remove supernatant. Repeat and re-suspend in a final column of 50µL 1 X NEB buffer 3.1.

18 Mix 10µL tungsten beads (again vortex whilst aliquoting to ensure beads are in suspension) with 8µL RNP complex.

Prepare equipment and materials for microparticle bombardment using the Biolistic particle delivery system PDS-1000/He (BioRad)

19 Sterilise the following components by soaking in 70% ethanol for 5 minutes in a sterile Petri dish:
Macrocarriers
Stopping screens
Rupture discs (1350 psi)

20 Allow to dry in a sterile hood.

21 Ensure PDS-1000/He has been set up according to manufacturer's instructions.

22 Clean inside of chamber and components (rupture disc retainer, microcarrier launch components and sample dish holder) with 70% IMS.

23 Prepare macrocarrier discs with previously prepared microcarriers (tungsten/RNP complex). Vortex and pipette 18µL into the centre of a macrocarrier trying to spread out into a small circle. Allow to dry for 1-2 hours (will not dry completely).

Microparticle bombardmentdelivery system PDS-1000/He (BioRad)

24 Rupture disc retainer assembly
1. Unscrew rupture disc retainer at top of the chamber.
2. Insert rupture disc (1350 psi) with sterile tweezers and ensure it is sitting securely in the retainer.
3. Screw back in the top of the chamber and gently tight with the torque wrench provided with the PDS-1000/He system.

25 Microparticle launch assembly
1. Unscrew metal ring/lid and place a sterile stopping screen at the bottom ensuring it fits securely.
2. Place macrocarrier into the holder (small metal disc with hole in centre) ensuring the RNP/tungsten is facing up, away from the metal.
3. Secure the macrocarrier into the holder using the red plastic tool provided.
4. Place macrocarrier onto the assembly with the RNP/tungsten facing towards the stopping screen.
5. Screw metal lid back on top of the assembly and slide the whole assembly onto the top shelf of the chamber.

26 Place agar plate with *Isochrysis galbana* cells onto the dish holder and slide onto the second from bottom shelf. Remove lid from plate and close the chamber door and secure.

Citation: Andrea Highfield, Glen Wheeler (06/21/2021). Microparticle delivery of CRISPR-Cas9 ribonucleoprotein to Isochrysis galbana for gene editing of URA3 gene.. https://dx.doi.org/10.17504/protocols.io.bvxen7je

27 Slowly turn on helium and adjust pressure to approximately 200psi greater that the burst pressure of the rupture disc.

28 Turn the pump attached the the chamber on and turn the central switch to VAC.

29 Allow vacuum pressure to reach 27 inHg and turn switch to HOLD.

30 Press FIRE switch immediately and hold down until you hear the pop of the rupture disc bursting and the helium pressure drops.

31 Turn the central switch to VENT to release the vacuum.

32 Open the bombardment chamber door and remove the plate and replace lid.

33 Protect plates from light for several hours then incubate plates under usual culture conditions.

Selection on semi-solid agarose plates

34 In advance, prepare and autoclave a stock of 2.1% low meting point (LMP) agarose in milliQ water for the semi-solid plates.

35 24 hours after microparticle bombardment wash the cells off the plates by gently pipetting with 3 mL 50% salinity F/2.

36 Transfer to microfuge tube and gently spin down at 2000rpm for 3 minutes. Remove supernatant and re-suspend in 1.5 mL 50% salinity F/2 (this will be sufficient for three selection plates).

37 Heat LMP agarose stock and keep molten at 65-90°C.

38 Into a 15 mL centrifuge tube, aliquot 9mL 50% salinity F/2 containing 5-fluoroorotic acid (5-FOA) to give a final concentration of 15 µgmL-1 and 50µg mL-1 uracil (final volume of plate is 10.5mL).

39 Add 1 mL molten LMP agarose to media and mix by inversion.

Citation: Andrea Highfield, Glen Wheeler (06/21/2021). Microparticle delivery of CRISPR-Cas9 ribonucleoprotein to Isochrysis galbana for gene editing of URA3 gene.. https://dx.doi.org/10.17504/protocols.io.bvxen7je

40 Add 500µL concentrated cells and mix by inversion.

41 Pour into 55 mm Petri dish and allow to cool and set (partially).

42 Carefully transfer up to 4 of the 55 mm plates (DO NOT INVERT) into a large square Petri dish (120 mm) and seal with Parafilm. Transfer to incubator.

43 Colonies become visible by eye after approximately three weeks. These colonies can be picked using a sterile pipette and the colony transferred to 1 mL 50% salinity F/2 in 24 well culture plates. We continued to use selection of 15 ugmL-1 5-FOA and also supplemented media with 50 µgmL-1 uracil.

Citation: Andrea Highfield, Glen Wheeler (06/21/2021). Microparticle delivery of CRISPR-Cas9 ribonucleoprotein to Isochrysis galbana for gene editing of URA3 gene.. https://dx.doi.org/10.17504/protocols.io.bvxen7je