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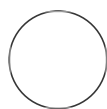
## 🌐 Prime Editing in *Physcomitrium patens*

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## ABSTRACT

PEG-mediated naked DNA protoplast transfection has been the standard transformation approach for more than thirty years in the moss *Physcomitrium patens*. The advent of the CRISPR-Cas technology has given a new opportunity for this approach in moss and other plants. Recently, the *Prime Editing*, a CRISPR-Cas9 based approach combining the efficiency of genome searching of the nCas9, its association with a Reverse Transcriptase and the encoding of desired edit in 3' of the guide RNA has allowed the specific edition of any base-pair in the vicinity of the *Streptococcus pyogenes* Cas9 PAM –NGG . We present here a complete procedure of *Prime Editing* in *P. patens* starting from the production of the optimal tissue to obtain protoplasts, followed by generation of protoplasts, their transfection using PEG-mediated naked DNA and their subsequent regeneration. Finally we describe two modes of selection based on the choice of edited gene target. The direct selection mode is dedicated to the selection of edits generating a loss of function of the *PpAPT* gene that can be selected directly after regeneration on 2-Fluoroadenine-containing medium. The indirect selection mode allows for the pre-selection of transfected protoplast with an antibiotic independently of the targeted gene, the screen for the proper edit being identified after genotyping of the selected plants.

## GUIDELINES

The present protocol is based on different procedures previously published (mainly Schaefer et al 1991, Cove et al. (2009) and Charlot et al. 2022). It contains four distinct sections: tissue production, protoplast production, protoplast transformation, and plant regeneration and selection.

## MATERIALS

If not mentioned otherwise, solutions are made using ultra pure water type milliQ or equivalent and is referred as mQ-H<sub>2</sub>O hereafter.

### I. Specific material

#### Cellophane disk

Cellophane disks can either be purchased as such or be cut into disk from a cellophane sheet or roll. In both case the disk diameter should be 8.5 cm, so they fit standard 9 cm Petri dish after re-hydration. Autoclave sterilization can be performed either in mQ-water, in which case they can be laid on medium directly, or dry condition in which case they need to be re-hydrated with sterile mQ-water before use.

### II. Stock solutions:

### **8.5% (w/v) D-mannitol**

D-mannitol	85 g
mQ-H <sub>2</sub> O	up to 1 l

Autoclave, store at room temperature

### **CaCl<sub>2</sub> 1M**

CaCl <sub>2</sub> ·6H <sub>2</sub> O	219 g
mQ-H <sub>2</sub> O	up to 1 l

Autoclave, store at room temperature

### **Driselase 2%**

Driselase powder	2g
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Driselase Plant tissue culture tested Merck MilliporeSigma (Sigma-Aldrich) Catalog #D8037

8.5% (w/v) D-mannitol	up to 100 ml
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Proceed as follow: add both components and stir the solution for 30 minutes at room temperature. Transfer unto 50 ml tube and centrifuge 20 minutes at 4500 g. Filter sterilize using a 0.22 µm filter the supernatant that should be clear light brown. Make 5 ml aliquot in sterile tubes and store at -20°C. Thaw 30 minutes before use.

### **MMM buffer**

MES	2 g (1%)
Mannitol	17 g (8.5%)
MgCl <sub>2</sub> ·6H <sub>2</sub> O	610 mg (15 mM)
mQ-H <sub>2</sub> O	180 ml

pH 5.6 with KOH and up to 200 ml with mQ-H<sub>2</sub>O and autoclave, store at room temperature.

### **PEG transformation solution**

Mannitol	7 g (0.38 M)
Ca(NO <sub>3</sub> ) <sub>2</sub> (H <sub>2</sub> O) <sub>4</sub>	2.36g (0.1M)

PEG 4000 (Serva,Roth) 40 g (40 % w/v)  
 Tris pH=8 1ml of 1M Tris pH=8.0  
 mQ-H<sub>2</sub>O up to 100 ml

Filter sterilize using 0.22 µm filter and make of 3 ml aliquot before storage at -20°C

### Phosphate-1000X Stock solution

KH<sub>2</sub>PO<sub>4</sub> 25g (184 mM)  
 mQ-H<sub>2</sub>O up to 80 ml

pH 7 with KOH, up to 100 ml with ddH<sub>2</sub>O and autoclave, store at 4°C.

### TES 1000X (Hoagland's A-Z trace element solution):

H3BO3	614 mg
MnCl2.4H2O	389 mg
Al2(SO4)3.K2SO4.24H2O	55 mg
CoCl2.6H2O	55 mg
CuSO4.5H2O	55 mg
ZnSO4.7H2O	55 mg
KBr	28 mg
KI	28 mg
LiCl	28 mg
SnCl2.2H2O	28 mg
H2O	up to 1 l

Autoclave, store at 4°C.

## III. Media

### Solid PPNH<sub>4</sub> medium

A	B	C
	Quantity	Final Concentration
Ca(NO <sub>3</sub> ) <sub>2</sub> 4H <sub>2</sub> O	0.8 g	4 mM
MgSO <sub>4</sub> 7H <sub>2</sub> O	0.25 g	1 mM
FeSO <sub>4</sub> 7H <sub>2</sub> O	12.5 g	45 µM
TES 1000X	1 ml	
Phosphate-1000X	1 ml	18.4 mM
Ammonium tartrate dibasic	0.5 g	2.7 mM
Agar, Plant tissue culture	7 g	0,7%
H <sub>2</sub> O dist.	up to 1l	

Autoclave, pour unto 9 ml Petri dishes and store at room temperature.

#### **Solid PPNH<sub>4</sub> medium + Mannitol + CaCl<sub>2</sub>**

A	B	C
	Quantity	Final Concentration
Ca(NO <sub>3</sub> ) <sub>2</sub> 4H <sub>2</sub> O	0.8 g	4 mM
MgSO <sub>4</sub> 7H <sub>2</sub> O	0.25 g	1 mM
FeSO <sub>4</sub> 7H <sub>2</sub> O	12.5 mg	45 µM
Micro-elements-1000X	1 ml	
Phosphate-1000X	1 ml	18.4 mM
Ammonium tartrate dibasic	0.5 g	2.7 mM
Mannitol	66 g	6.6 %
CaCl <sub>2</sub>	6 ml	6 mM
Agar, Plant tissue culture	7 g	0,7%

A	B	C
H <sub>2</sub> O dist.	up to 1l	

Autoclave, pour into 9 ml Petri dishes and store at room temperature.

### **Alginate solution**

A	B	C
	Quantity	Final Concentration
Mannitol	8.5 g	8.5 %
Alginate	1.5 g	1.5 %
ddH <sub>2</sub> O	100 ml	

Ensure that the alginate is mostly dissolved by heating at 65°C. prior autoclaving. Then, autoclave and filter with a 0.22 µm filter and make 50 ml aliquot . Store at room temperature.

## **IV. Antibiotic stock solutions**

### **G418 1000X**

G418      300 mg  
mQ-H<sub>2</sub>O   10 ml

Filter with a 0.22 µm filter and make 1 ml aliquot . Store at -20°C

### **Hygromycin B 1000X**

Hygromycin B      250 mg  
mQ-H<sub>2</sub>O            10 ml

Filter with a 0.22 µm filter and make 1 ml aliquot . Store at -20°C

### **2-Fluoroadenine (2-FA) 1000X (10 mM)**

2-FA      15.3 mg  
DMSO    10 ml

Store at -20°C

## BEFORE START INSTRUCTIONS

The protocol described hereafter needs to be performed at room temperature (<22°C) if not mentioned otherwise and in a sterile environment, e.g. vertical or horizontal laminar flow hood. Standard *in vitro* plant culture precautions concerning sterility (e.g. surface cleaning, reagent filtering and autoclaving) are required. The different solutions used for this protocol can and should be prepped before starting the procedure so to not lose time during its execution. Note that two components of the transfection protocol, the driselase 2% and the PEG transformation solution, are stored at -20°C, therefore before starting the procedure, bring them back to room temperature. The small metallic material (spatula and twizzer) can be sterilized just before use by flaming or using hot glass or metallic beads. Finally, for an optimum protoplast regeneration and protonemata growth, Petri dish with vent must be used and they must be sealed with 3M Micropore tape (or equivalent) to allow normal gas exchange in the plate (no parafilm).

- 1 Using a binocular lens, check carefully the starting *P. patens* material for an eventual contamination (bacteria, fungus). The tissue must be green. An axenic culture is essential before performing any further experimental step.

10m

## *P. patens* protonema culture

1w



- 2 Collect the tissue either from a seven day old protonemal culture or from spot inoculum with mixed tissue (protonema and gametophore) in a sterile 50 ml tube containing 20 ml sterile mQ-water. 2m
- 3 Using an homogenizer, grind the tissue into 5- 15 cells small fragments and filtrate the ground material through a sterilized 140 µm sieve. Recover the fragment left on the sieve with a sterile spatula and transfer it in a new 50 ml tube containing sterile mQ-water, adapt the water volume to the amount of recovered tissue. 1m
- 4 Inoculate 2 ml of the freshly fragmented protonema per Petri dish poured with solid PpNH<sub>4</sub> medium overlaid with a sterilized cellophane disk. Seal the plates with 3M Micropore tape. 1m
- 5 Transfer the plate(s) in a incubator for seven days in standard plant growth condition (Temperature 22°C, light conditions: 16h light/ 8h darkness, 70 µE.m<sup>-2</sup>.sec<sup>-1</sup>). 1w
- 6 To obtain an homogenous protonemal culture necessary for the protoplast production, the repetition of the step 1-5 twice. These repetitions allow for the elimination of any gametophore tissue that will not protoplast and allow for the amplification of the tissue. Typically, one seven-days old entrained protonemal plate can be used to inoculate eight new plates. 2w

## *P. patens* protoplast production 2h

- 7 Harvest and transfer rapidly the protonemal tissue from four 6-7 days old cultures plates from Step 6 into 15 ml of 8.5% mannitol. Add 5 ml of Driselase 2% from frozen stock for a final Driselase concentration of 0.5% and incubate 40 minutes at room temperature with occasional gentle plate swirling.

### Note

With this protocol, protoplast yield should be between half million to one million protoplasts per 9 cm Petri dish culture.

- 8 Filter the suspension through 80-100 µm sieve and let incubate at room temperature for 10 minutes.

- 9 Filter the suspension to 40-50 µm sieve and pour the flow-through (containing the isolated protoplasts) into a tube that can withstand low speed centrifugation (e.g. 50 ml conical tube).
- 10 Centrifuge 5 minutes at 150 x g to sediment the protoplasts.
- 11 Living protoplasts will sediment to form a dark green pellet. Discard the supernatant by tilting the tube and gently resuspended the pellet into 20 ml of mannitol 8.5% supplemented with 10 mM CaCl<sub>2</sub> final.
- 12 Centrifuge 5 minutes at 150 x g to sediment the protoplasts.
- 13 Living protoplasts will sediment to form a dark green pellet. Discard the supernatant by tilting the tube and gently resuspended the pellet into 20 ml of mannitol 8.5% supplemented with 10 mM CaCl<sub>2</sub> final. Evaluate the number of obtained protoplasts using a Malassez counting chamber or equivalent.
- 14 Centrifuge 5 minutes at 150 x g to sediment the protoplasts. Discard the supernatant by tilting the tube. Proceed directly to the transfection procedure.

### ***P. patens* protoplast transfection and plating**

- 15 In a 15 ml sterile tube, put 15 µg of total vector DNA necessary for the *Prime Editing* procedure resuspended in sterile mQ-water in a maximum of 30 µl volume. This DNA can be produced by standard PCR amplification or plasmid prep amplification. The two vectors necessary are the expression vector for the Prime editor and the vector expressing the pegRNA specific to the target of choice. In addition a third vector containing an antibiotic resistance cassette must be added in case of indirect selection of transfectants. 1m
- 16 Add 300 µl of protoplast resuspended in MMM buffer to the vector DNA. Protoplast 1m

concentration in the MMM buffer can be anywhere between  $1.2 \times 10^6$  and  $1.6 \times 10^6$  without affecting transformation efficiency with the volume ratio for each component presented in this protocol.

- 17** Add 300  $\mu$ l of PEG transformation solution (from frozen stock) and mix gently (do not vortex or pipet up and down the suspension) but thoroughly, to produce a visually uniform solution. 1m
- 18** Incubate the tube for 5 minutes in a 44°C water bath. 5m
- 19** Let the tube stand at room temperature for 10 minutes. 10m
- 20** The PEG transformation solution is toxic to the protoplasts. Dilute the transformation mix by adding, at one minute interval, five times 300  $\mu$ l, and then five times 1 ml of mannitol 8.5% supplemented with 10 mM  $\text{CaCl}_2$  final (6.5 ml total). Mix gently by swirling the tube after each dilution step. 10m
- 21** Let the tube stand at room temperature for 30 minutes. At this stage protoplasts have been transformed and are ready to be plated.
- 22** To concentrate the protoplast suspension, centrifuge 5 minutes at 150 x g to sediment the protoplasts. Discard the supernatant and add up to 4 ml of fresh mannitol 8.5% supplemented with 6 mM  $\text{CaCl}_2$  final for one transformation tube. 5m
- 23** Add 4 ml of alginate solution to the protoplast suspension and mix gently by pipetting up and down. 30s
- 24** Pour readily 2 ml of mixture per 9 cm Petri dish filled of solid regeneration medium overlayed with sterile cellophane. Each standard transfection is spread on four 9 cm Petri dishes. 30s

- 25 Let the plates stand for 30 minutes to one hour in the laminar flow hood (covered and sealed) before transferring the plate to the incubator (Temperature 22°C, light conditions: 16h light/ 8h darkness, 70  $\mu\text{E} \cdot \text{m}^{-2} \cdot \text{sec}^{-1}$ ). 30m
- 26 Let the protoplasts grow for 6 to 7 days on the transformation plates. Observe the plates for the number of divided protoplasts since this is the actual number that count to evaluate transformation efficiency if necessary. After a week of growth, most of what can regenerate will have and you can identify them as plant with cells number between 2 and 15 cells 1w

## Transfected *P. patens* plant selection 4w

- 27 Selection mode is dependent of the edited target. If the edited target can be selected positively, e.g. the non edited plant dies on the selection medium and the edited plant survives as it is the case for endogenous gene *PpAPT* and the selection compound 2-Fluoroadenine (2-FA) use the *Plant direct selection* case. If the locus phenotype cannot be directly selected or generate an unknown phenotype used the *Plant indirect selection* case.

Step 27 includes a Step case.

### Plant direct selection

step case

### Plant direct selection

To use when the gene *PpAPT* is targeted and yields *apt* variant resistant to 2-FA.

- 28 Transfer the cellophane containing the regenerating protoplasts onto PpNH<sub>4</sub> plate supplemented with 10  $\mu\text{M}$  2-FA and place the plates for at least a week in standard plant growth condition (Temperature 22°C, light conditions: 16h light/ 8h darkness, 70  $\mu\text{E} \cdot \text{m}^{-2} \cdot \text{sec}^{-1}$ ). During this step, only the plants that have the gene *PpAPT* disrupted will grow as 2-FA is lethal to the wild-type strain.

- 29 The selected plants are now ready for tissue amplification on standard PpNH<sub>4</sub> medium and subsequent genotyping according to the specific target edition in the gene *PpAPT*.

Step 29 includes a Step case.

### Plant indirect selection

step case

### Plant indirect selection

To use when the phenotype generated by the edit is not selectable directly or unknown

- 30** Transfer the cellophane containing the regenerating protoplasts onto solid PpNH<sub>4</sub> medium plate supplemented with the appropriate antibiotic (for which resistance is present in the transformation vector). Incubate plates for at least a week in standard plant growth condition (Temperature 22°C, light conditions: 16h light/ 8h darkness, 70 μE.m<sup>-2</sup>.sec<sup>-1</sup>). During this step most of the plants should die, leaving 50 to 250 growing plants.
- 31** Under a binocular lens, isolate individual antibiotic resistant plant using sterile twizzer and transfer them unto fresh PpNH<sub>4</sub> medium and incubate plates for at least a two weeks in standard plant growth condition (Temperature 22°C, light conditions: 16h light/ 8h darkness, 70 μE.m<sup>-2</sup>.sec<sup>-1</sup>). At this point selected plant are ready for genotyping and identification of the edit event.

2w