Version 2
Dec 28, 2020

Transformation of *Diplonema papillatum* by electroporation V.2

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1 Works for me dx.doi.org/10.17504/protocols.io.bq3nmyme

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

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ABSTRACT

Variant protocol for transformation of *Diplonema papillatum* by electroporation using a "home-made" transformation buffer. The procedure was devised based on previously published protocols by Kaur *et al.* (DOI: 10.1111/1462-2920.14041) and Dyer *et al.* (DOI: 10.3791/54342). For additional details, see also Faktorová *et al.* (DOI: 10.1111/1462-2920.15130).

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Faktorová D, Kaur B, Valach M, Graf L, Benz C, Burger G, Lukeš J. 2020. Targeted integration by homologous recombination enables in situ tagging and replacement of genes in the marine microeukaryote Diplonema papillatum. Environ. Microbiol. 22:3660–3670. (https://doi.org/10.1111/1462-2920.15130)

DOI

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PROTOCOL CITATION

Matus Valach 2020. Transformation of Diplonema papillatum by electroporation. **protocols.io** https://dx.doi.org/10.17504/protocols.io.bq3nmyme
Version created by Matus Valach

MANUSCRIPT CITATION please remember to cite the following publication along with this protocol

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KEYWORDS

Diplonema, transformation, selection, protist, electroporation, antibiotic resistance, diplonemid

CREATED

Dec 28, 2020

LAST MODIFIED

Dec 28, 2020

PROTOCOL INTEGER ID

45902

MATERIALS TEXT

MATERIALS

Sodium Phosphate monobasic **Contributed by users**

Glucose **Contributed by users**

KCl **Contributed by users**

CaCl2 **Contributed by users**

MgCl2 **Contributed by users**

BSA **Contributed by users**

Sucrose **Contributed by users**

HEPES **Contributed by users**

EDTA **Contributed by users**

Inosine triphosphate **Contributed by users**

G418 (geneticin sulfate) **Bioshop Catalog #GEN418**

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BEFORE STARTING

Perform a simple test of antibiotic resistance of wild-type cells in the chosen culture conditions, e.g., temperature (16 *vs* 20 *vs* 27 °C), medium composition (e.g., horse serum *vs* fetal bovine serum) or antibiotic supplier. Into a 24-well plate, distribute 1.5 mL medium per well and add the antibiotic at several different concentrations (e.g., for G418, choose 0, 50, 75, 100, 150, and 200 µg/mL). This arrangement (6 columns, each with a different antibiotic concentration) allows to perform three WT replicates together with one positive control resistant to the antibiotic of choice. Inoculate 1–5×10^5 cells per well and let the cells grow for 3–4 days, then examine the extent of growth. The lowest antibiotic concentration at which the WT cells do not grow is then used for the selection.
For example, when cultivating *Diplonema papillatum* in a horse serum-based medium and using G418 (*Bioshop*; potency min. 650 µg/mg), 100 µg/mL is the threshold value at 20 °C, but >125 µg/mL is needed for efficient selection at 16 °C.

1 Prepare the transformation (cytomix-like) buffer.

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A	B
Component	**Final concentration**
HEPES pH7.5	25 mM
KCl	25 mM
CaCl2	0.15
NaH2PO4 pH7.5	10
MgCl2	2.5
EDTA	1
glucose	30 mM (0.5%)
sucrose	145 mM (4.35%)
bovine serum albumin (BSA)	0.1 mg/mL
inosine triphosphate (ITP) [or hypoxanthine]	1 mM

The addition of ITP (or hypoxanthine) is optional. If preparing a large volume of the buffer, make aliquots and store them at −70 °C until further use.

2 Inoculate *Diplonema* cells at 1–2×10^5 /mL into 100 mL OSS medium and let them grow for 2–3 days.

The resulting amount of cells is usually sufficient for 3–4 transformations. Therefore, if performing additional transformations, scale-up the cultivation volume.
If performing the selection at 20 °C, pre-culture is done at this same temperature.

3 Harvest the cells while they are in the exponential phase (optimal density 2–4×10^6 /mL). Wash twice with OS (i.e., medium without the serum) and aliquot the cells into tubes, so that after the final centrifugation, each pellet contains 2×10^7 to 10^8 cells. Remove as much OS buffer as possible. Keep the cells on ice.

The number of cells required depends on the pulse parameters (see the step #7 below). For high voltage conditions, 10^8 cells means that more cells will survive the pulse and the probability of a successful transformation increases. For low voltage conditions, 2×10^7 cells per transformation is preferable because at 10^8 cells, in general, a much higher proportion (up to 95%!) of transformants has the transformation DNA construct inserted at a non-homologous location. However, if the homologous integration is of little interest, using the higher amount of cells can be beneficial since many more clonal cell lines can be obtained.

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4 Resuspend the pellet in ice-cold 200 µL transformation buffer (see the recipe above), immediately centrifuge (4 °C, 1,000×g, 2 min), and discard the supernatant.

5 Resuspend the pellet in ice-cold 100 µL transformation buffer supplemented with 0.5–2 µg linearized DNA (e.g., a PCR product or a restriction fragment of a plasmid).

Optimally, add the DNA in a volume of 5 µL or less. To the negative control, add the same volume of the buffer used to solubilize the linearized DNA (e.g., 10 mM Tris pH8.0).

6 Immediately transfer the cell suspension into an electroporation cuvette (0.2 mm), which has been pre-cooled on ice.

7 Wipe the cuvette to remove moisture, quickly transfer the cuvette into an electroporation apparatus (e.g., *Gene Pulser Xcell* from *Bio-Rad*), and apply the pulse.

Pulse parameters:
1. 1,500 V, 0.4 ms (also referred to here at "**high voltage**"); or
2. 140 V, 1,400 µF ("**low voltage**").

Clone selection is more straightforward and clear-cut for the option **1** (**high voltage**) and we observed that a higher proportion of clones has had the construct integrated at the intended locus (~80%), but the number of clones is limited (up to 5 independent cell lines have been obtained, but usually only ~2 clones).
In constrast, cell survival is much more substantial in the option **2** (**low voltage**) and may be preferred when numerous clones are required (up to 45 independent clones have been obtained).

8 Immediately after the pulse, put the cuvette back on ice, add 1 mL cold (5–10 °C) OSS, and resuspend the cells.

9 Transfer the cell suspension into a well of a 24-well (or 48-well) plate. Distribute the pulsed cell suspension into 12–48 wells (depending on the expected or desired number of independent clones; e.g., 12 is recommended for high voltage conditions, while 48 for low voltage conditions). Add additional OSS into each well (~0.8 and ~0.4 mL when using 24- and 48-well plates, respectively). Cultivate for 5–8 h without selection.

10 Prepare OSS with the antibiotic of choice at a concentration that is double of the selection concentration (e.g., 200 µg/mL G418 if the final selection concentration is to be 100 µg/mL). To each well with pulsed cell suspension in OSS, add an equal volume of this medium. The final volume is usually 1.6–2 mL (24-well plates) or 0.8–1 mL (48-well plates).

Make sure that the final concentration of the antibiotic is as determined by the resistance test.
Optionally, keep a single well without the antibiotic (i.e., add an equal volume of just OSS) to keep track of the recovery of the pulsed cells. This is especially useful when applying a high voltage pulse to less than 5×10^7 cells.

Let the cells grow for 4–7 days. Observe the cells in the plates under a microscope to check their growth. If there is

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11 visible growth (i.e., cells swimming in the 'medium column'), transfer an aliquot of the cells into a new plate with a 1.5–2× higher concentration of the antibiotic (e.g., if using G418 at 100 µg/mL, this cell passaging should be done at 150–200 µg/mL). After a growth for additional 4–7 days, start analyzing the clones or make conserves for a later analysis.

Passaging the cells through a medium with a higher concentration of the antibiotic ensures that only truly resistant clones (i.e., those expressing the antibiotic resistance-confering gene at a sufficient level) are selected. Optionally, perform 10× serial dilutions of cells from each selection well into a new plate with fresh medium to ensure that truly independent clones are selected. This phase may take up to 3 weeks in total.

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