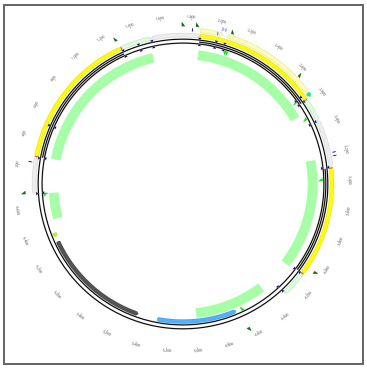

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Transformation of Diplonema papillatum by electroporation V.3

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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).

MATERIALS

MATERIALS

- Sodium Phosphate monobasic
- Glucose
- KCl
- CaCl2
- MgCl2
- BSA
- Sucrose
- HEPES
- EDTA
- Inosine triphosphate
- G418 (geneticin sulfate) **Bioshop Catalog #GEN418**

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

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BEFORE START INSTRUCTIONS

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.

	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%)

	A	B
	bovine serum albumin (BSA)	0.1 mg/mL
	inosine triphosphate (ITP) [or hypoxanthine]	1 mM

Note

The addition of ITP (or hypoxanthine) is optional (alternatively, ATP can be used). 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 supplemented with 0.05% tryptone and let them grow for 2–3 days.

Note

Cell density is ~5-times higher when cultivating *Diplonema* cells in a medium containing 0.05% tryptone compared to a medium without such supplementation. This improves survival after the pulse and is especially useful for **high-voltage** conditions (see below), which seems to favorise homologous integration. The resulting amount of cells is usually sufficient for 4–6 transformations. Therefore, if performing additional transformations, scale-up the cultivation volume. (If performing selection at 20 °C, pre-culture is done at this same temperature.)

3 Harvest the cells while they are in the late exponential phase (optimal density 8×10^6–2×10^7 /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 1–4×10^8 cells. Remove as much OS buffer as possible. Keep the cells on ice.

Note

The number of cells required depends on the pulse parameters (see the step #7 below). For high voltage conditions, 4×10^8 cells means that more cells will survive the pulse and the probability of a successful transformation increases. For low voltage conditions, much lower number of cells per transformation should be used because higher cell densities generally result in a quasi-totality of transformants having the transformed DNA construct inserted at non-homologous locations. Conversely, if homologous integration is of little interest, subjecting a high number of cells to the pulse is beneficial since many more clonal cell lines can be obtained.

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 1–4 µg linearized DNA (e.g., a PCR product or a restriction fragment of a plasmid).

Note

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**").

Note

Cell line selection is more straightforward and clear-cut for the option **1** (**high voltage**) and we observed that a higher proportion of transformants has had the construct integrated at the intended locus (~60%), but the number of independent cell lines is limited (up to 5 independent cell lines have been obtained, but usually only about 2).
In contrast, 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 cell lines have been obtained). However, as indicated above, transformants tend to integrate the construct at a non-homologous location much more prominently.

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 24–48 wells (depending on the expected or desired number of independent clones, but the higher the number of wells, the more likely it is that a pure clonal cell line will quickly be obtained). Add additional OSS into each well (~1 and ~0.5 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).

Note

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.

11 Let the cells grow for ~2 days. Observe the cells in the plates under a microscope to check their growth. If there is visible growth, i.e., cells swimming in the 'column', transfer an aliquot of these swimming 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 well population passaging should be done at 150–200 µg/mL). After a growth for additional 5–9 days, start analyzing well populations or make conserves for later analyses.

Note

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-conferring gene at a sufficient level) are selected.

12 If a well population is a mixture of cells containing a wild-type allele and a correctly integrated DNA construct, perform 10× serial dilutions of cells from each selection well into a new plate with fresh medium (if using G418, usually at 100–150 µg/mL) to ensure that truly independent cell lines are selected. This phase may take up to 3 weeks in total.