Chapter 1

Genetic Manipulation of *Paradiplonema papillatum*

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Abstract

Here we present a collection of methods established for genetic manipulation of *Paradiplonema papillatum* (also referred to as *Diplonema papillatum)*, so far the only diplonemid species that is genetically tractable and can therefore serve as a model organism, enabling functional studies of this very abundant and species-rich group of marine protists. We describe a complete transformation protocol which includes: (1) the Alamar blue assay, a method that serves to identify antibiotics, to which *P. papillatum* is sensitive and can be used for transformant selection; (2) the design and preparation of DNA constructs for functional genomics; and (3) two protocols for electroporation and transformant screening. We conclude with an immunoprecipitation protocol optimized for pulling down target-tagged proteins along with their interaction partners, allowing the characterization of protein complexes.

Key words Diplonemids, *Paradiplonema papillatum*, *Diplonema papillatum*, Marine micro-eukaryotes, Genetic manipulation, Model organism, Transformation, DNA construct, Selection marker, Homologous recombination

1 Introduction

Diplonemids belong to the most abundant and diverse heterotrophic micro-eukaryotes in the world's oceans [1–5]. Despite their ecological significance, our understanding of these unicellular flagellates has been limited, largely due to the absence of a model species. To shed light on the evolutionary and ecological roles of these major players in the oceanic ecosystem, we have recently decided to turn at least one diplonemid species into a genetically tractable system, which is a critical step toward functional studies of its genes. To establish a new model system, three steps of transformation road map [6] must be fulfilled simultaneously: (1) identification of suitable selectable markers, (2) design of an integratable and functional construct, and last, but not least (3) a method to deliver into the cell an exogenous DNA that would be transcribed into an mRNA and translated into a functional protein product.

Paul A.M. Michels et al. (eds.), *Euglenozoa: Methods and Protocols, Volume 2*, Methods in Molecular Biology, vol. 3014,
https://doi.org/10.1007/978-1-0716-5146-9_1,

Here, we describe several procedures that allow genetic manipulations of the diplonemid type species, *Paradiplonema* (also called *Diplonema*) *papillatum*. It can be easily and cheaply axenically cultured, reaches high density, and grows in large volumes [7]. The addition of this species to the 22 other established model organisms expands considerably the diversity represented among marine model protists [6].

First, we identified several antibiotics to which *P. papillatum* is sensitive and which are therefore suitable as selectable markers [7]; the detailed protocol is described below. Second, we designed constructs encoding a selected antibiotic resistance marker, gene tag, and additional genomic sequences from *P. papillatum* to facilitate vector integration into chromosomes. After initial attempts [7], we found out that the targeted integration was only successful after homology arms were extended to 1.0–2.0 kb [8, 9], an observation that was confirmed in several follow-up studies [10–13]. Lastly, we adapted established electroporation conditions widely used in diplonemid's sister clade, the kinetoplastids, specifically *Trypanosoma brucei* [14], and we demonstrated that electroporated constructs stably integrate in the *P. papillatum* nuclear genome.

The now established basic toolkit for reverse genetics described in detail in this chapter enables functional studies in the gene-rich *P. papillatum* [15], and has the potential for expansion into other diplonemid species available in culture [9, 16].

2 Materials

2.1 Determination of Resistance to Antibiotics Using Alamar Blue Assay

Chemicals and equipment needed:

1. 0.125 mg/mL resazurin sodium salt in 1 × PBS, filter-sterilized.
2. 1 × PBS.
3. Antibiotic stock solutions used for this assay: G418 (15 mg/mL), hygromycin B (50 mg/mL), phleomycin (100 mg/mL), puromycin (4 mg/mL), blasticidin S (10 mg/mL), zeocin (80 mg/mL), tetracycline (1 mg/mL).
4. 96-well plates.
5. Sterile basins (for multichannel-pipette buffer storage).
6. Multichannel pipettes.
7. Humidified box.
8. Cell culture incubator (settings: 15–27 °C; *see* **Note 1**).
9. A device for measuring fluorescence (e.g., Tecan Infinite M200).

2.2 Design and Preparation of DNA Constructs for Electroporation

2.2.1 Original Protocol (Lukeš Lab)

1. Tissue culture fume hood (or a bench with natural gas supply and a Bunsen burner).
2. Nitrile gloves.
3. Ethanol-resistant fine-lined marker.
4. Refrigerator (4 °C), freezer (−20 °C).
5. 70% ethanol spray for disinfection, household bleach for decontamination.
6. Pipettes and compatible pipette tips.
7. Cell culture incubator (settings: 37 °C).
8. PCR machine.
9. 0.2 μL PCR tubes, sterile.
10. 1.5 mL centrifugation tubes, sterile.
11. PCR primers.
12. *P. papillatum* genomic DNA.
13. pDP series plasmid of choice.
14. High fidelity DNA polymerase (recommended: Phusion or Q5 polymerase, NEB).
15. 3 M sodium acetate, pH 5.2, 96% and ice-cold 70% ethanol.
16. Centrifuge, 4–22 °C, up to 20,000 × *g* (for 1.5-mL tubes).
17. MilliQ water.

2.2.2 Modified Protocol Using Cloning of Homology Arms into the Plasmids (Burger Lab)

1. Cell culture incubator (settings: 37 °C).
2. PCR machine.
3. 0.2 μL PCR tubes, sterile.
4. 1.5 mL centrifugation tubes, sterile.
5. PCR primers.
6. pDP series plasmid of choice.
7. Restriction enzymes of choice (e.g., *Eco*RI, *BamH*I, *Hind*III, *Ssp*I) and their required buffers.
8. NEBuilder master mix (NEB).
9. Chemically (or electroporation) competent *Escherichia coli* DH5α (or similar strain).
10. LB plates with 100 μg/μL ampicillin (for *E. coli* transformation).
11. Liquid LB medium with 100 μg/μL ampicillin (for colony screening via plasmid isolation).
12. Bacterial plasmid isolation kit (e.g., Monarch® Spin Plasmid Miniprep Kit, NEB; *see* **Note 2**).
13. Precipitation buffer (1.75 M NaCl, 70 mM Tris-HCl, pH 8.0).
14. Isopropanol.
15. Centrifuge, 4–22 °C, up to 20,000 × *g* (for 1.5 mL tubes).

16. 80% ethanol.
17. 10 mM Tris–HCl, pH 8.0.

2.2.3 Modified Protocol Using Cloning of Homology Arms into the Plasmid, a Method Based on pBA3294 and pBA3295 Plasmids (Akiyoshi Lab)

1. pBA3294 (TY-YFP-TY, NeoR) and pBA3295 (TY-tdTomato-TY, hygromycin) plasmids—available upon request to Bungo Akiyoshi for now, later they will be deposited to Addgene. Plasmid sequences are included here [17].
2. Restriction enzymes of choice (e.g., *Pac*I, *Asc*I, *Not*I) and their required buffers.
3. DNA ligase.
4. KOD one DNA polymerase (Merck).
5. PCR machine.
6. 0.2 μL PCR tubes, sterile.
7. 1.5 mL centrifugation tubes, sterile.
8. PCR primers.
9. Chemically (or electroporation) competent *E. coli* DH5α (or similar strain).
10. LB plates with 100 μg/μL ampicillin (for *E. coli* transformation).
11. Liquid LB medium with 100 μg/μL ampicillin (for colony screening via plasmid isolation).
12. Bacterial plasmid isolation kit (e.g., Qiagen miniprep).
13. Centrifuge, room temperature (for 1.5 mL tubes).
14. 100% and 70% ethanol.

2.3 Electroporation and Isolation of Transformants

2.3.1 Original Protocol (Lukeš Lab)

1. Tissue culture fume hood
2. Nitrile gloves.
3. Ethanol-resistant fine-lined marker.
4. Refrigerator (4 °C), freezer (−20 °C).
5. 70% ethanol spray for disinfection, household bleach for decontamination.
6. Pipettes and compatible pipette tips, sterile.
7. Cell culture incubator (settings: 10–25 °C, optimally at 16–22 °C).
8. Vented cell culture flasks (e.g., 25 cm^2 or larger).
9. Inverted microscope with 10–40 × objectives.
10. Microscopic glass slides and cover glasses.
11. Hemocytometer.
12. Ice and styrofoam icebox.
13. Medium for *P. papillatum* (*see* Volume 1 - Chapter 1, “Isolation and cultivation of diplonemids”).

14. 50 mL and 1.5 mL centrifugation tubes, sterile.
15. 2 mm electroporation cuvettes, sterile.
16. 24-well or 48-well cell culture plates, sterile.
17. 25 cm^2 culture flasks, sterile.
18. Electroporation instrument (e.g., AMAXA Nucleofector II).
19. Centrifuge, 4–22 °C, up to 2000 × *g*.
20. Antibiotic of choice (e.g., G418, hygromycin, puromycin).

2.3.2 Alternative Protocol (Burger Lab)

1. Tissue culture fume hood.
2. Cell culture incubator (settings: 10–25 °C, optimally at 16–22 °C).
3. Vented cell culture flasks (e.g., 25 cm^2 or larger).
4. Inverted microscope with 10–40 × objectives.
5. Microscopic glass slides and cover glasses.
6. Hemocytometer.
7. Ice and styrofoam icebox.
8. Cytomix-like buffer (25 mM HEPES, pH 7.5, 25 mM KCl, 0.15 mM $CaCl_2$, 10 mM NaH_2PO_4, pH 7.5, 2.5 mM $MgCl_2$, 1 mM EDTA, 30 mM (0.5%) glucose, 145 mM (4.35%) sucrose, 0.1 mg/mL bovine serum albumin (BSA), 1 mM inosine triphosphate (ITP) [or adenosine triphosphate (ATP)]; *see* **Note 3**).
9. OSS medium (artificial sea water, 1% horse serum) supplemented with 0.05% tryptone.
10. OS medium.
11. 50 mL and 1.5 mL centrifugation tubes, sterile.
12. 2 mm electroporation cuvettes, sterile.
13. 24-well or 48-well cell culture plates, sterile.
14. 25 cm^2 culture flasks, sterile.
15. Electroporation instrument (e.g., Gene Pulser Xcell; *Bio-Rad*).
16. Centrifuge, 4–22 °C, up to 2000 × *g*.
17. Antibiotic of choice (e.g., G418, hygromycin, puromycin).

2.4 Transformants Screening

1. Kit for isolation of genomic DNA—e.g., Qiagen DNA isolation kit (Qiagen) or Exgene Cell SV (Bohemia Genetics).
2. High fidelity DNA polymerase—e.g., Phusion or Q5 polymerase (NEB Biolabs).

2.4.1 Original Protocol (Lukeš Lab)

Genomic DNA Isolation, PCR Using Genomic DNA

RNA Isolation, cDNA Synthesis, and RT-PCR

1. TriReagent (MRC).
2. Chloroform, isopropanol, 75% ethanol.
3. RNAse-free water.
4. QuantiTect Reverse Transcription Kit (Qiagen).
5. DNA polymerase —e.g., OneTaq polymerase (NEB Biolabs).
6. Specific primers.

Western Blot (Immunoblot) Analysis

1. 2 × SDS sample buffer.
2. Bolt 4%–12% Bis-Tris polyacrylamide gels (Invitrogen) or 4–12% (v/v) NuPAGE gels (Invitrogen, NP0322BOX).
3. PVDF membrane.
4. 5% (w/v) non-fat milk prepared in PBS, 0.5% (v/v) Tween 20.
5. Specific primary antibody (at desired concentration), secondary antibody conjugated with horseradish peroxidase (1:1000) (Sigma), and an antibody that will be used as a loading control (e.g., monoclonal anti-a-tubulin antibody produced in mouse (1:1000) (Sigma, T9026).
6. Clarity western ECL substrate (Bio-Rad).

2.4.2 Alternative Protocol for Transformant Screening: Single-Step DNA and RNA Isolation (Burger Lab)

1. Refrigerator (4 °C), freezer (−20 °C).
2. 1.5 mL centrifugation tubes, sterile.
3. Centrifuge, 4–22 °C, up to 20,000 × *g*.
4. Vortex.
5. Ice and styrofoam icebox.
6. "Home-made" Trizol substitute (38% phenol [saturated, pH 4.3 Liq.]), 0.8 M guanidine thiocyanate, 0.4 M ammonium thiocyanate, 0.1 M sodium acetate pH 5, 5% glycerol; *see* **Note 4**).
7. Chloroform.
8. Isopropanol.
9. 95–100% ethanol.
10. 70% ethanol.
11. 10 mg/mL glycogen (or amylopectin), RNase-free.

2.5 Immuno-precipitation

1. Refrigerator (4 °C).
2. Centrifuge, 4–25 °C, up to 20,000 × *g*.
3. Magnetic stand.
4. 1.5 mL centrifugation tubes.
5. 30-gauge needle.

6. 1 × complete EDTA-free protease inhibitors (Sigma, 11873580001).
7. Rotator for incubation of the beads in the fridge.
8. IPP150 buffer: 10 mM Tris-HCl, pH 6.8, 150 mM NaCl, 0.1% IGEPAL CA-630; Sigma I8896).
9. CHAPS buffer: 20 mM HEPES, pH 7.4, 100 mM NaCl, 0.5% CHAPS (Roche 10810118001).
10. For Protein A (PrA) IP: IgG Sepharose 6 Fastbeads (GE Healthcare, 52–2083-00 AH); 0.1 M glycine, pH 3.0; 1 M Tris–HCl, pH 9.0.
11. For V5 IP: V5-Trap magnetic Particles M-270 (Chromotek, v5td-20).

3 Methods

3.1 Determination of Resistance to Antibiotics Using the Alamar Blue Assay

1. To find out the appropriate antibiotics concentration used for selection following the transformation of *P. papillatum* (*see* Table 1 and **Notes 5** and **6**), use the Alamar blue assay as we described in [18, 19] to determine the EC_{50} value, i.e., the inhibitor concentration at which 50% of the cell population is affected (*see* **Note 7**).
2. Use a 96-well plate and prepare triplicates for each drug. Use a sterile basin for the liquids and a multichannel pipette for more convenient pipetting of triplicates.
3. Prepare sterile 1 × PBS (or medium) and add 200 μL to the wells around the chamber to prevent the plate from drying out.
4. Prepare the antibiotic stocks (G418, hygromycin B, phleomycin, puromycin, blasticidin S, zeocin, and tetracycline) and test

Table 1
Tested resistance markers and their concentration suited for *P. papillatum* selection

Antibiotic	Stock conc. (mg/mL)	dilution	Final conc. (μg/mL)
Hygromycin	50	1:400	125
G418	15	1:200	75
Phleomycin	100	1:200	500
Puromycin	4	1:200	20
Blasticidin	10	1:200	50
Tetracycline	1		*see* **Note 5**
Zeocin	80		*see* **Note 6**

each drug concentration in triplicate using their serial dilutions at the desired cultivation temperature (15–27 °C; *see* **Note 1**) for 48 h. Use a well without the drug at the end of each row as a control.

5. Pipette 16 μL of the antibiotics and 184 μL of the medium into the first well (first column)—the total volume is 200 μL.
6. Pipette 100 μL of the cell medium into the remaining wells in the columns.
7. Make serial dilutions of the drug (100 μL) across the plate (to obtain a set of 10 × dilutions of the drug in each column). The last row without the drugs serves as a negative control.
8. Count the cells and adjust the density to a desired concentration (for *Paradiplonema* 2×10^5 cells/mL). Add 100 μL of cells to each well. The final cell concentration is 1×10^5 cells/mL, and the final cell number is 2×10^4 cells per well.
9. Incubate the multi-well plates for 48 h at the desired cultivation temperature in a humidity-controlled container (the duration may be adjusted if necessary).
10. For the colorimetric evaluation of assay, add 20 μL of the resazurine dye.
11. Incubate for 24 h at the appropriate temperature in a humidified box (*see* **Note 8**).
12. The fluorescence of inner wells with samples and negative controls is measured, e.g., with a Tecan Infinite M200 instrument, using an excitation wavelength of 540–570 nm (peak at 544 nm) and an emission wavelength of 580–610 nm (peak at 590 nm). Use the Fluorescent intensity mode, and the following settings: excitation: 544 nm, width: 9 nm; emission: 590 nm, width 20 nm; mode—bottom; gain—optimal; plate-type: Costa 96-well flat transparent.
13. Analyze the Alamar blue assay data with appropriate software, e.g., GraphPad Prism (GraphPad), using nonlinear regression (curve fit) and a sigmoidal dose-response analysis to generate EC_{50} curves and derive the EC_{50} value of individual tested antibiotics from the curve.

3.2 Design and Preparation of DNA Constructs for Electroporation

Here we present three alternative protocols that were developed independently in our laboratories that are equally working and recommended (*see* **Note 9**).

3.2.1 Original Protocol (Lukeš Lab)

This protocol was developed at the beginning and is described in detail in [8], briefly:

1. The transformation cassettes were originally designed and prepared by a fusion PCR approach using high-fidelity DNA polymerase (e.g., Phusion or Q5). The approach and details are depicted in Fig. S2 in [8].
2. The first three individual PCRs are used to amplify (i) 5′ long homology region—upstream of the STOP codon (PCR A), (ii) the cassette designed to replace and/or tag a gene of interest (PCR B), and (iii) the 3′ long homology region—downstream of the STOP codon (PCR C). The PCR B-Fw and PCR B-Rv primers overlap with PCR A-Rv and PCR C-Fw primers, respectively (*see* **Note 10**).
3. Nested primers (PCR D-Fw and Rv) are used for joining all three pieces in the final product (PCR D). For details, see Experimental procedures in [8].
4. Amplified PCR products need to be gel-purified (using, e.g., GeneAll Expin Combo GP purification kit) and ethanol-precipitated. The DNA is then resuspended in 10–20 μL of milli-Q water and stored in −80 °C.

3.2.2 Alternative Protocol Using Cloning of Homology Arms into the Plasmids (Burger Lab)

Before Starting the Procedure

For C-terminal tagging, any restriction enzyme (RE) site that occurs exclusively in the region between the unique *Not*I and *BamH*I sites of a pDP plasmid (namely, pDP002–pDP010 and pDP013–pDP028) can be used to cut the plasmid in order to insert the upstream arm: *Not*I, *Spe*I, *Sma*I, *Cla*I, and *Eco*RI. Since *Eco*RI is the most practical enzyme from an economic viewpoint, it is usually the preferred option, and the examples below reflect this. The downstream arm is inserted into the unique *Hind*III site, located just downstream of the 3′ untranslated region of the neomycin resistance gene.

To prepare the DNA construct for the transformation of *P. papillatum*, the generated plasmid will need to be partitioned into two segments: the bacterial vector and the tagging construct to be knocked in the *P. papillatum* genome. This is ideally done with a single RE, which does not occur anywhere in the constant portion of the tagging plasmid (i.e., the region between the *BamH*I and *Hind*III sites for C-terminal tagging or the region between *Not*I and *BamH*I sites for N-terminal tagging), while ensuring that this chosen enzyme's sites occur in the regions of both upstream and downstream homology arms. In the absence of any such suitable RE site in one (or both) construct arms, the missing one (or two) sites can be added to the ends of the homology arms as part of the primer (i.e., into the forward primer for the upstream arm and into the reverse primer for the downstream arm). For reasons stated above, the usual choice is *Eco*RI, unless an *Eco*RI site occurs in the target region. In these latter cases, any RE whose site is not present in the target region can be selected. *Ssp*I is a common and safe option due to its site being AT-rich, which means that: (i) it has low

frequency in *P. papillatum* coding sequences and (ii) it only marginally increases the melting temperature of the amplification primers.

In this procedure, the NEBuilder® system (NEB) is used to generate the target tagging plasmid. This requires overlaps of 15–25 bp between each pair of DNA fragments that are to be assembled. The constant overlapping segments of the primers listed below are 17–21 bp-long and have been used successfully numerous times (*see* **Note 11**).

Plasmid Design and Construction

1. The design procedure starts from the target genomic region. Aim to select homologous arms of >1.0 kb, with the optimal size range being 1.5–2.0 kb. Although even as short segments as 750 bp can work, shorter arms require increased numbers of transformations to obtain a correctly integrated knock-in cell line. Avoid regions with repeats, especially close to the termini of homology arms. Also examine restriction enzyme sites around the potential homology arms.
2. Based on the considerations mentioned above, design primers for the upstream homology arm (upArm) amplification.

```
upArm_fwd = AAGTGCTGGACATCGG—{no site / EcoRI / SspI / etc.}—
upArm_sequence
upArm1_rev = ACCTGCGCTTCCAGGGGATCC—reverse_complement_u-
pArm_sequence => for pDP015/pDP025 and their derivatives
upArm2_rev = GACCCGGAGCCGCTGCC—reverse_complement_upArm_se-
quence => alternative for pDP006/pDP022 and their derivatives
```

3. Based on the considerations mentioned above, design primers for the downstream homology arm (dnArm) amplification.

```
dnArm_fwd = TTACGTGCTGCAAGTTT—dnArm_sequence
dnArm_rev = TAACAATTTCACCAAAGAA—{no site / EcoRI / SspI /
etc.}—reverse_complement_dnArm_sequence
```

4. Digest the selected plasmid with *EcoR*I + *BamH*I + *Hind*III, then column-purify the reaction and determine its DNA concentration.
5. PCR amplify the upArm and dnArm segments from genomic DNA of *P. papillatum*, then column-purify the reactions and determine their DNA concentration.
6. Assemble a NEBuilder reaction by combining the 2 × NEBuilder master mix, digested plasmid, upArm PCR product, and dnArm PCR product following the recommendations. If the assembly can be performed in a PCR machine with a heated cover to avoid evaporation, the reaction can be safely downscaled up to 4 times (from the manufacturer-recommended 20 μL down to 5 μL).

7. Transform chemically competent *E. coli* using the entire NEBuilder reaction and plate on LB medium with ampicillin.
8. Screen 3–6 colonies by isolating plasmids from 1 mL bacterial cultures (LB + ampicillin) and digesting them with suitable REs. (Usually, all colonies have the expected plasmid, but sequencing the entire plasmid is highly recommended.)
9. Isolate a larger amount of plasmid DNA (>10 μg is recommended, usually 2–3 mL of bacterial culture) from the colony that contains the correctly assembled and sequence-verified plasmid.
10. Use the selected RE to digest the tagging construct. For example, in 50 μL combine: 5 μg plasmid, 2–3 μL RE, 5 μL 10 × RE buffer, and complete with deionized water. Digest for 3–18 hours at 37 °C, then heat-inactivate for 20 min at 65–80 °C (or as recommended by the RE manufacturer).
11. The digested DNA can be either column-purified or precipitated. The latter is the preferred option because it minimizes material loss. To a 50 μL digestion, add 325 μL of the precipitation buffer, mix well, then add 525 μL isopropanol and mix again. Incubate 3–18 h at 4 °C, then centrifuge (30 min, >15,000 × *g*, 4 °C). Remove supernatant while avoiding the minute pellet. Wash with 80% ethanol for 5 min at room temperature, then re-centrifuge and remove the supernatant. Resuspend in 10 μL of 10 mM Tris-HCl, pH 8.0. Proceed to *P. papillatum* transformation (see below).
12. To verify the integration in the target locus and the tagged allele expression, extract DNA and RNA (see below).
13. To verify the integration, use primers that bind upstream of the upArm and downstream of the dnArm to cover the entire locus. If the targeted region is too large (>7.0 kb), amplification of the entire locus may be problematic, and it may be necessary to perform two PCRs: (i) using a forward primer that binds upstream of the upArm and a reverse primer binding in the tag or resistance gene, (ii) using a reverse primer that binds downstream of the dnArm and a forward primer binding in the tag or resistance gene.

3.2.3 Alternative Protocol Using Cloning of Homology Arms and pBA3294 and pBA3295 Plasmids (Akiyoshi Lab)

Plasmid Design and Construction

pBA3294 and pBA3295 are synthetic plasmids that allow YFP- or tdTomato-tagging of the gene of interest at the endogenous locus in *P. papillatum* [17]. The design of these vectors is based on pEnT series used for *T. brucei* [20]. For C-terminal tagging, use *Pac*I and *Asc*I REs to insert two ~2.0 kb homology arms containing one unique site (e.g., that recognized by *Not*I): one corresponding to the 3′ UTR (starting just after its stop codon) with *Pac*I and *Not*I, and one corresponding to the 3′ region of this gene (ending just before its stop codon) with *Not*I and *Asc*I. After cutting the

fragments with respective REs, the two DNA fragments are ligated into pBA3294 or pBA3295 cut with *Pac*I and *Asc*I. Screen colonies by Nanopore whole plasmid sequencing. Make sure that the two 2.0 kb-long DNA fragments do not have any *Pac*I or *Asc*I site. Otherwise, you will need to clone the fragments using a restriction-free method (e.g., Gibson assembly). If *Not*I is not unique, use other REs (e.g., *Sbf*I, *Afl*II). For N-terminal YFP tagging *Xba*I and *BamH*I can be used to insert two homology arms with one unique restriction site (e.g., *Not*I). It may be necessary to test primers of different lengths to obtain the desired PCR products.

An example of C-terminal tagging using classic cloning with *Pac*I/*Asc*I and *Not*I sites: DIPPA_26288.mRNA.1 (Histone H3.1)

To amplify 2.0 kb-long 3′ UTR DNA fragment from genomic DNA (referred to as "3′UTR"), use

- 3′UTR-forward primer with *Pac*I: gatc TTAATTAA acaaaatctgttccctttttggacttttttccag
- 3′UTR-reverse primer with *Not*I: gatc GCGGCCGC gcgccgatggc-gatggtg

To amplify 2.0 kb-long DNA fragment corresponding to the C-terminal end of the coding sequence ("CDS3"), use

- CDS3′-Forward primer with *Not*I: gatc GCGGCCGC tactgcg-tatgctggtatgg
- CDS3′-Reverse primer with *Asc*I and a GlyAlaGly linker (make sure that the coding sequence is in frame with the YFP/tdTomato tag): gatc GGCGCGCC c ACCTGCTCC caaaaggtcgccacggatg

1. PCR amplify the two fragments from genomic DNA using KOD one DNA polymerase.
2. Perform PCR purification using a PCR purification kit.
3. Digest the PCR products using appropriate restriction enzymes. Digest the pBA3294 or pBA3295 vector using *Pac*I and *Asc*I.
4. Run on agarose gel and perform gel extraction.
5. Ligate the two DNA fragments into pBA3294 or pBA3295 using a classic ligation reaction.
6. Use ligated products to transform *E. coli*.
7. Isolate plasmids by miniprep kit.
8. Screen 2 colonies by whole plasmid sequencing.
9. Digest 5–10 μg DNA by *Not*I for 1 h.

10. Add 2 × volume of 100% ethanol.
11. Spin down at 21,000 × *g* for 5 min.
12. Discard supernatant.
13. Add 360 μL of 70% ethanol.
14. Spin down at 21,000 × *g* for 5 min.
15. Discard supernatant carefully in a safety cabinet.
16. Dry for 10 min.
17. Add 20 μL of transfection reagent.
18. Proceed to *P. papillatum* transformation (see below).

3.3 Electroporation and Obtaining of the Transformants

Here we present two alternative protocols that were developed independently in our laboratories that are equally working and recommended (*see* **Note 9**).

3.3.1 Original Protocol (Lukeš' Lab)

1. Transform *P. papillatum* cells by electroporation (see **Note 9**).
2. Count the cells and use 5×10^7 cells for a single electroporation.
3. Harvest the cells by centrifugation (1300 × *g*, 5 min, room temperature) (*see* **Note 12**).
4. Resuspend the cell pellet in 100 μL of AMAXA Human T-cell solution (combine 81.8 μL of Human T-cell nucleofactor solution with 18.2 μL Supplement).
5. Add the cell suspension to 2.0–5.0 μg of DNA resuspended in 10 μL of milliQ. Do not add any DNA for the negative control.
6. Put the final solution into the cuvette, close the cap and place it in the electroporator.
7. Execute program X-001.
8. Subsequently transfer the suspension to a flask with 10 mL of *Diplonema* medium with the supplied disposable pipette.
9. Place the flasks in an incubator at 27 °C and let them grow for about 24 h.
10. Take out the flasks after 24 h and distribute their contents into 24-well plates.
11. Add 1.5 mL of electroporated cells in the first row (6 wells) of 24-well plates (9 mL total). Add the corresponding amount of drugs into the first row (row A). Add 1 mL of the medium (including selectable drug) in the rows B and C and 500 μL in the row D.
12. Mix the contents of the first well with a pipette and transfer 500 μL to row B to make the dilution and so on till the end of the column (*see* **Note 13**).

13. For the positive control, add 10 mL of fresh medium without antibiotics to the rest of the cells in the flasks with electroporated cells.
14. Selection usually takes 2–6 weeks depending on the selection drug.

3.3.2 Alternative Protocol (Burger Lab)

1. Inoculate *P. papillatum* at 1–2 × 10^5 cells/mL into 100 mL OSS medium supplemented with 0.05% tryptone and let them grow for 2–3 days (*see* **Note 14**).
2. Harvest the cells while they are in the late exponential phase (optimal density 8 × 10^6–2 × 10^7/mL) by centrifugation (2000 × *g*, 5 min, 4 °C) (*see* **Note 15**).
3. Resuspend the pelleted cells in OS (i.e., medium without the serum), then transfer them into 1.5 mL tubes and centrifuge (2000 × *g*, 5 min, 4 °C). For example, if harvesting cells from 100 mL of OSS, resuspend the cell pellet in 2–3 mL OS and transfer them into two 1.5 mL tubes.
4. Repeat the washing with OS once more, then aliquot the cells into tubes, so that after the final centrifugation, each pellet contains 1–4 × 10^8 cells. Remove as much OS as possible. From this point on, keep the cells on ice.
5. Resuspend the pellet in ice-cold 200 μL transformation buffer, immediately centrifuge (4 °C, 1000 × *g*, 1 min), and discard the supernatant. If not proceeding to electroporation immediately, keep the cells on ice without any buffer. Note that any delay decreases transformation efficiency, but a pause of ≤5 min can be accommodated if necessary.
6. Resuspend the pellet in ice-cold 100 μL Cytomix-like transformation buffer supplemented with 1–4 μg linearized DNA. Optimally, add the DNA in 5 μL or in an even lower volume. To the negative control, add the same volume of the buffer used to solubilize the linearized DNA (e.g., 10 mM Tris-HCl, pH 8.0).
7. Transfer the cell suspension immediately into a precooled electroporation cuvette.
8. Wipe the cuvette to remove moisture, transfer it quickly into the electroporation apparatus (e.g., Gene Pulser Xcell from *Bio-Rad*), and apply the pulse. Pulse parameters: (A) 1500 V, 0.4 ms (also referred to here at "high voltage") or (B) 140 V, 1400 μF ("low voltage") (*see* **Note 16**). Immediately after the pulse, put the cuvette back on ice, add 1 mL of cold (1–5 °C) OSS, and resuspend the cells.
9. As quickly as possible, transfer the pulsed cell suspension into a single well of a 24-well (or 48-well) plate, then distribute the suspension into all wells of the plate (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 to allow the cells to recover.

10. Prepare OSS with the antibiotic of choice at a concentration that is double 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 antibiotic-supplemented medium. The final volume in a well is then 2 mL (24-well plates) or 1 mL (48-well plates) (*see* **Note 17**).
12. Let the cells grow for 2–4 days and observe them in the plates under a microscope to check their growth. If there is a visible growth, i.e., cells actively swim (as opposed to passive floating), 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 passaging of a well population 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 (*see* **Note 18**).
13. 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. Expect this phase to take 1–3 weeks.

3.4 Transformants Screening

Here we present two alternative protocols that are equally working and recommended (*see* **Note 9**).

3.4.1 Original Protocol (Lukeš Lab)

The following techniques are used to verify that the transgene is: (1) correctly integrated in the genomic DNA (Genomic DNA isolation and PCR using genomic DNA), (2) properly transcribed (RNA isolation, cDNA synthesis, and RT-PCR) and translated (Western blot—Immunoblot analysis).

Genomic DNA Isolation and PCR Using Genomic DNA

1. After completing selection, make a ~20 mL exponential culture of the cell lines and isolate genomic DNA (e.g., using Qiagen DNA isolation kit or Exgene Cell SV).
2. Culture transformants for up to 8 weeks prior to testing by PCR to make sure that they contain a stably integrated construct.
3. Use specific primers to verify by PCR the correct integration in the genome (e.g., PCR A-Fw and PCR B-Rv, see Design and

preparation of DNA constructs for electroporation, Subheading 3.2.1). Confirm construct integration in the genome by sequencing of the PCR products.

RNA Isolation, cDNA Synthesis, and RT-PCR

1. To isolate total RNA, spin ~10^8 cells (2000 × *g*, 5 min) and resuspend the pellet thoroughly in 1 mL of TriReagent.
2. Leave it at room temperature for 5 min to dissociate nucleoprotein complexes. (At this step, the suspension can be stored in −80 °C.)
3. Add 0.2 mL of chloroform used and shake the tube vigorously for 15 s, leave at room temperature for 2–15 min, then spin (12,000 × *g*, 15 min, 4 °C).
4. Transfer upper aqueous phase to a fresh tube, add 500 μL of isopropanol, incubate for 10 min at room temperature, and spin afterwards (12,000 × *g*, 10 min, 4 °C). Remove the supernatant.
5. Add 1 mL of 75% ethanol, shake for 5 s, and spin down (7500 × *g*, 5 min, 4 °C). Remove the supernatant.
6. Let the sample dry on the bench for 5–15 min (lying tube), dissolve the RNA in about 20 μL of RNAse-free water (can be warmed up to 60 °C).
7. Measure the RNA concentration using Nanodrop.
8. Store RNA in −80 °C.
9. Prepare cDNA using QuantiTect Reverse Transcription Kit with random primers.
10. For RT-PCR, perform PCR on cDNA with specific primers and PCR-grade DNA polymerase (e.g., OneTaq).
11. Prepare the same reactions without reverse transcriptase (RT-) to serve as a negative control.

Western Blot (Immunoblot) Analysis

1. Prepare cell lysates in 2 × SDS sample buffer using 5 × 10^6 cells per lane.
2. Separate the samples on a 4–12% (v/v) SDS-PAGE gel.
3. Transfer the proteins subsequently onto the PVDF membrane by electroblotting.
4. Block the membranes with 5% (w/v) non-fat milk prepared in PBS 0.5% (v/v) Tween 20.
5. Probe them with the specific primary antibodies at 4 °C overnight.
6. Wash the membrane 5 × for 5 min in 1 × PBS-Tween.
7. Subsequently incubate the membrane with the secondary antibody of choice.

8. Use a chemiluminescence substrate of choice for band visualization.
9. Use a loading control—an antibody against a protein with high and ubiquitous expression (e.g., tubulin)—to verify even loading across all the wells.
10. Select positive cell lines with the correct size of the tagged protein. They can be further used for an immunofluorescence assay to check the tagged protein localization or for an immunoprecipitation assay to pull-down the protein's interaction partners.

3.4.2 Alternative Protocol for Transformant Screening: Single-Step DNA and RNA Isolation (Burger Lab)

1. Add 5 volumes of the "home-made" Trizol substitute to the sample, *see* **Note 19**. (When working in 1.5 mL tubes, for practical reasons, the minimal and maximal volume of the sample is 20 and 180 μL, respectively.)
2. Vortex vigorously for 10–30 s (depending on the viscosity and protein and nucleic acid concentration of the sample).
3. Incubate at room temperature for 3–5 min.
4. Add 1 volume of chloroform (relative to the original volume of the sample) and mix vigorously 10–20 times by inverting the tube. Do not vortex.
5. Incubate at room temperature for 3–5 min.
6. Spin (>12,000 × *g*, 10–15 min, 4 °C), depending on the viscosity and protein and nucleic acid concentration of the sample.
7. Transfer the upper aqueous phase into a new tube. Avoid the white DNA precipitate at the interphase. (If the sample volume was 100 μL, the aqueous phase should be ~450 μL).
8. Add 1.1 volume of isopropanol, relative to the volume of the aqueous phase. If low RNA content is expected in the sample, prior to isopropanol, add 10–30 μg of glycogen (or amylopectin) to facilitate precipitating nucleic acids and spotting the pellet. Recommended final concentration of glycogen is ~50–150 μg/mL.
9. Mix well by inverting the tube and incubate 20–60 min (on ice, at 4 °C or at room temperature).
10. Spin (>12,000 × *g*, 20–45 min, 4 °C) and discard the supernatant (*see* **Note 20**).
11. Add 1 volume of 70% (aq.) ethanol (relative to the volume of the aqueous phase) and mix gently. Ensure that the pellet is submerged in the solution and not sticking to the tube wall.
12. Incubate 5–10 min at room temperature.
13. Spin for (>12,000 × *g*, 5 min, 4 °C) and discard the supernatant.

14. Let the pellet dry at room temperature, so that no traces of 70% ethanol remain. Drying under vacuum is not recommended because of over-drying that makes it harder to dissolve the pellet.
15. Add RNase-free water and let the pellet completely dissolve (*see* **Note 21**).

3.5 Immunoprecipitation

1. For cell lines tagged by Protein A (PrA) or V5 tag, prepare 5×10^8 exponentially growing cells in *Diplonema* medium supplemented with selection antibiotic(s) (*see* **Note 22**).
2. Harvest cells by centrifugation at 1000 × *g* for 10 min and subsequently resuspend the pellet in 1 mL of either ice-cold IPP150 or CHAPS buffers, supplemented with 1 × complete EDTA-free protease inhibitors.
3. Pass the pellet 5 × through a 30-gauge needle.
4. Subsequently, clear the lysate twice by centrifugation (12,000 × *g*, 10 min, 4 °C).
5. Incubate the cleared supernatant with 75 μL of IgG Sepharose 6 Fastbeads (for Protein A tagged cell lines) or 15 μL of V5-Trap magnetic Particles M-270 (for V5 tagged cell lines) on an orbital shaker at 4 °C for 2–3 h to enable binding of the tagged protein.
6. Wash the beads 5 × using the cell lysis buffer, the last two washes of beads are performed using a buffer without detergent.
7. Then either (1) elute the PrA-tagged protein together with its potential interaction partners using 100 μL of 0.1 M glycine, pH 3.0, by rotating for 5 min at room temperature and immediately neutralizing with 10 μL of 1 M Tris-HCl, pH 9.0, or (2) remove the supernatant and keep the V5-trapped magnetic beads.
8. Store the samples (elution samples or beads) in −80 °C.
9. Process aliquots of the input, flow-through and elution/beads fractions, and assess fractionation efficiency by immunoblotting.
10. The eluates or beads are ultimately analyzed by mass spectrometry.

4 Notes

1. *P. papillatum* propagates best in the range of 16–22 °C. In the upper range (22–27 °C), cells still grow and divide but are heat-stressed (>28 °C will lead to growth arrest and cell death). In the lower range (4–16 °C), cells divide more slowly than at

higher temperatures. Temperature and composition of the medium will ultimately result in physiological changes, e.g., accumulation of storage lipids, changes in membrane composition, rate of mitochondrial metabolism, or the relative and absolute content of some proteins. Therefore, the optimal growth conditions for a particular experiment should be determined beforehand in a small-scale setup.

2. Screening can be done via precipitation, but for *P. papillatum* transformation, column-purified plasmids are critical. For regular screening of minipreps, it is convenient and economical to use regenerated (up to ~10 times) silica columns following the protocol devised by [21].
3. If preparing a large volume of the buffer, make aliquots and store them at −70 °C until further use.
4. Stored in a glass bottle protected from light (e.g., wrapped in aluminum foil) and kept at 4 °C, the "home-made" Trizol substitute solution is stable for at least 3 months. Discard if pink or purple coloration appears, which is indicative of phenol oxidation.
5. Tetracycline at 40 μg/mL is not lethal for *P. papillatum*.
6. Zeocin at 3.2 mg/mL is not lethal for *P. papillatum*.
7. Alternatively (in case of no access to resazurin), 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 wild-type 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 wild-type cells do not grow is then used for selection. For example, when cultivating *P. papillatum* in the OSS 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.
8. The cells can be cultivated with resazurin for a shorter or longer period based on the color of the cultures in the wells (bright pink color means that the cells are alive, while dark blue indicates that the cells do not have any metabolic activity/are dead, so to obtain the biggest color changes is beneficial for getting precise values. Also, it is important to maintain the same treatment duration for all your replicates.

9. Three alternative protocols of "Design and preparation of DNA constructs for electroporation" and two of "Electroporation and obtaining of the transformants" and "Transformant screening" are presented. All protocols were developed independently in our laboratories and are equally working and recommended based on equipment availabilities of the various laboratories.
10. The length of homologous arms depends on the length of the non-repetitive sequence in the surrounding of the genes and can vary from 1.0 to 2.0 kb.
11. The tagging plasmid can also be constructed via serial RE digestion and DNA ligation, but this procedure is slower and has a limited throughput.
12. Make sure that you have labelled flasks with medium at hand before electroporation.
13. So far, we tested the following antibiotics for selection: puromycin (N-terminal tagging using DF_Dp_01 construct [7]), G418 (C-terminal PrA-tagging using pDP002 plasmid [8]), hygromycin (C-terminal V5-tagging using pDP011 plasmid [8]), and transfectants were subjected to selection with increasing concentrations of puromycin (12–40 μg/mL) for $Puro^R$, G418 (25–80 μg/mL) for Neo^R, or hygromycin (150–275 μg/mL) for Hyg^R containing constructs.
14. Cell density is ~5 × higher when cultivating *P. papillatum* 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 favor homologous integration. The resulting number of cells from a 100 mL culture 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.)
15. The number of cells required depends on the pulse parameters (see below). For high voltage conditions, 4×10^8 cells means that more cells will survive the pulse, and thus the probability of a successful transformation increases. For low voltage conditions, a 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 loci. 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 (e.g., when inserting a library of fragments).

16. Cell line selection is more straightforward and clear-cut for option A (high voltage) and leads to a higher yield of transformants with 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 option B (low voltage) and may be preferred when numerous clones are required (up to 45 independent cell lines have been obtained). However, as indicated above, such transformants predominantly integrate the construct at a non-homologous location.
17. Make sure that the final concentration of the antibiotic is the one determined during the resistance test. Optionally, keep a single well without the antibiotic (i.e., add an equal volume of 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.
18. 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) will be selected.
19. Compared to the commercial Trizol® procedure, this protocol (modified from [22]) makes use of generally available chemicals without the need for columns, thus allowing concurrent extraction of transcripts of all sizes, including RNAs <200 nt. It has been tested on diverse types of samples, especially protists, their organelles (e.g., mitochondria, plastids, ribosomes), and subcellular fractions. The procedure is scalable. Its yield, as tested with spiked-in radioactively labelled transcripts of 60–900 nt, is 70–90% (in general, increasing with length of the RNA). The minimal amount of RNA that could be reproducibly extracted was 10 ng (at a concentration of 200 pg/μL). Note that RNA extracted using this protocol is usually not DNA-free, so DNase treatment is necessary if pure RNA is required. Notably, circular DNA molecules up to ~9.0 kb are isolated at high yields.
20. For **steps 9** and **10**, longer incubation times and lower temperatures increase RNA recovery, especially from samples with low RNA concentration.
21. The yield of nuclear DNA and mRNA from 2×10^6–2×10^7 *P. papillatum* (1–2 mL of a late exponential-phase culture) is sufficient for at least 10 genomic screening PCR and a similar number of RT-PCR experiments.
22. For all IP experiments, use three replicates of each sample for mass spectrometry, with wild-type cells serving as a control.

Acknowledgments

This work was supported by the Czech Science Foundation (grant 25-15298S to J.L.), the Fonds de Recherche du Québec—Nature et Technologies (FRQNT; grant 2018-PR-206806 to G.B.) and the Natural Sciences and Engineering Research Council of Canada (NSERC; grants RGPIN-2014–05286 and RGPIN-2019–04024 to G.B.), and by the Wellcome Discovery Award (227243/Z/23/Z to B.A.).

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