

Transient Expression in Arabidopsis Mesophyll Protoplasts

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For citing this protocol:

Yoo, S.D., Cho, Y.H., and Sheen, J. 2007. *Arabidopsis* mesophyll protoplasts: A versatile cell system for transient gene expression analysis. *Nature Protocols*, 2: 1565-1575.

Sheen, J. 2002, A transient expression assay using *Arabidopsis* mesophyll protoplasts.

<http://genetics.mgh.harvard.edu/sheenweb/>

The protocol has been streamlined and can be applied to different types of plant materials. The growth condition of the plants seems most critical for experimental reproducibility. Each lab may need to work out the best plant growth conditions based on the available facilities and local supplies, including water, soil, air and light bulbs. Lower light (50-75 $\mu\text{mole m}^{-2} \text{s}^{-1}$) and shorter photoperiod (<13 hr) are more desirable to prolong vegetative growth. Clean air, water and soil are essential!! No need for excess nutrient or seedling transplant (sow your cold stratified seeds directly on wet soil). The quality of DNA and PEG is critical. The protocol is simple and the approach is powerful. However, it only awards success to the scientists who are willing to take the time needed to master the assay with patience and faith. Potential wounding and stress problems could be minimized during the experimental process to avoid high background. The protoplasts generated using this protocol have been used to study hormone, sugar, stress and defense responses using reporter genes that show similar responses in intact plants. Real-time RT-PCR is routinely used to confirm that the endogenous gene response is similar to that of the corresponding reporter gene. Good luck with your experiments!

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Updated on 9/26/97, 2/28/98, 9/11/00, 12/16/00, 4/6/01, 9/1/01, 9/29/01, 10/3/01, 4/21/02, 5/27/02, 10/10/03, 12/23/03, 3/22/04, 4/14/09, 9/9/09, 11/15/09, 6/17/10, 5/22/11, 9/16/2012

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A Transient Expression Assay Using *Arabidopsis* Mesophyll Protoplasts

A. Protoplast Isolation

Plant Materials:

BE plants grown on the B5 medium

Greenhouse-grown BE, Col, *Ler* and C24 plants and most mutants are fine

Use well expanded leaves from 3-4 weeks old plants (the second and/or third/fourth pair, 1-2 cm) before flowering. It's OK to use plants that are 1-3 weeks old but more plants are needed.

Suggested plant growth condition: low light (50-75 $\mu\text{mole m}^{-2} \text{s}^{-1}$), short photoperiod (12-13h), 22-25°C, water as needed (no need to add nutrient solution when grown in soil), good/fresh air flow (very important). Please check the local water and soil quality or contamination.

Protoplast Isolation Procedure:

Cut 0.5-1 mm leaf strips with fresh razor blades without wounding. This is perhaps the most tedious part for most people. Piling leaves for more efficient cutting is optional. However, I consider it easier and more efficient than peeling the lower epidermis of the leaves one by one. It takes some practice. Some labs use a tape to peel the epidermal layer and it seems to work well. A large preparation yields around 10^7 protoplasts/g fresh weight (about 100 to 150 leaves digested in 40-60 ml of enzyme solution). For a practice and most experiments, 10-20 leaves

digested in 5-10 ml cellulase/macerozyme solution will give $0.5 - 1 \times 10^6$ protoplasts that are enough for more than 50-100 samples ($1-2 \times 10^4$ protoplasts per sample). Please note that it is not necessary to use 10^6 protoplasts per sample for gene expression analysis as commonly recommended in other protoplast protocols. The experiments can be easily scaled up or down as long as the recommended DNA/protoplast ratio is followed (see below). To observe subcellular localization or protein-protein interactions by BiFC of tagged proteins, $10^3 - 10^4$ cells are more than enough. For promoter analysis, 100-1000 cells are sufficient, depending on the promoter strength and reporter gene used. For protein expression and co-immunoprecipitation, $10^4 - 10^5$ protoplasts are needed. Around 10^5 protoplasts are needed for RNA isolation.

Submerge the leaf strips in enzyme solution in a Petri dish, and put it into to a vacuum desiccator. Apply vacuum infiltration for 5-30 min. Continue the digestion for another 60 to 90 min with gentle shaking (40 rpm on a platform shaker) or digest up to 3 h without shaking in the dark (depending on the experimental goals and desirable responses). This step needs to be tested empirically for your own assay. For soil-grown plants, do not digest the leaves for overnight. The usual prolonged incubation of leaves for 12-18 h in the dark for protoplast isolation is stressful and might eliminate physiological responses of leaf cells. However, the stress might be potentially important for the dedifferentiation and regeneration processes when sterile plants grown on culture medium are used. The enzyme solution should turn green which indicates the release of round protoplasts (check under microscope, the size of Arabidopsis mesophyll protoplasts is around 15 to 50 μm). Release the protoplasts by shaking at 80 rpm for 1-5 min. No intention is made to release protoplasts 100%. Be gentle with the protoplasts!

Filter the enzyme solution containing protoplasts with a 35-75 μm nylon mesh. Spin at 100x g to pellet the protoplasts in a round-bottomed tube for 1-2 min (speed 3-4 with an IEC clinical centrifuge or <1000 rpm on a bench top centrifuge). Higher speed or the addition of CaCl_2 (50 mM) may be used if the protoplast recovery is poor. The pelleted protoplasts should be resuspended easily by gentle shaking. Wash protoplasts once in cold washing/incubation (WI) solution for electroporation or W5 solution for PEG transfection, and resuspend protoplasts in the same solution at $2 \times 10^5/\text{ml}$. Keep the protoplasts on ice (30 min) in WI or W5 solution unless cold responses will be studied. For some experiments, protoplasts could be kept at room temperature before use (Please test). Although the protoplasts can be kept on ice for at least 24 h, freshly prepared protoplasts should be used for the study of regulated gene expression, signal transduction, and protein trafficking, processing and localization. We use these protoplasts to study leaf cell responses to sugars, auxin, ABA, cytokinin, ethylene, GA, heat, cold, EtOH, H_2O_2 , calcium, elicitors and peptides. The responses in protoplasts are similar to those observed in intact plant leaves. These protoplasts are also a good source for the isolation of intact nuclei and chloroplasts. If W5 solution is used, spin down protoplasts (speed 3 for 1 min) and resuspend in MMg solution ($2 \times 10^5/\text{ml}$) before PEG transfection. For FACS (fluorescence-activated cell sorting) with protoplasts, it is better to use W5 instead of WI because the low solubility of mannitol.

B. PEG Transfection

All steps are carried out at 23°C

Add 10 µl DNA (10-20 µg of plasmid DNA of 5 kb in size. Please test down to 2-4 µg)

Add 100 µl protoplasts to a microfuge tube (2 x 10⁴ protoplasts), mix well

Add 110 µl of PEG/Ca solution, mix well (handle 6 samples each time)

Incubate at 23°C for 3-30 min (please test)

Dilute with 0.44 ml W5 solution, mix well

Spin at speed 3 (1 Krpm) in a Clinical centrifuge for 1 min, remove PEG

Resuspend protoplasts gently, dilute in 100 µl, add to 1 ml WI or W5 (6-well plates)

This procedure can be scaled up or down depending on the experimental needs

Due to the “transient” nature of the experiments, it is not necessary to perform experiments under sterile conditions. The addition of Amp (50 µg/ml) can prevent bacterial growth if it is necessary.

The system is most suitable for the study of early events in diverse signal transduction pathways, gene regulation, and cell death (Asai et al., *Plant Cell* 12:1823-1835, 2000).

The use of carrier DNA is unnecessary.

Use 6-well tissue culture dishes (Falcon 3046) for protoplast incubation. We now also use 12-well (0.5 ml) or 24-well (0.25 ml) plates for large experiments (with 50-200 samples).

The dish can be coated with 5% calf serum for 1 sec before use to prevent the sticking of the protoplasts to the plastic.

The protoplast incubation time is 2-6 h for RNA analysis and 2-16 h for enzyme activity analysis and protein labeling or immunoblot analysis. About 100-1000 protoplasts are sufficient for reporter enzyme assays, 10⁴-10⁵ protoplasts are required for protein labeling &

immunoprecipitation or immunoblot analysis, and 10⁵-10⁶ protoplasts for RNA analysis.

These protoplasts can be cultured for cell wall regeneration and cell cycle initiation with proper medium and plant hormones (Damm, B. et al., *MGG* 217: 6-12. 1989).

PEG transformation efficiency is 50-90% based on GFP expression. If your protoplasts are healthy (from proper leaf materials. Please test), most protoplasts remain intact.

Electroporation efficiency is 10-30% (depending on plant conditions). More than 50% protoplasts can be killed by electroporation. However conditions could be adjusted to reduce killing (Please test). Protoplasts produced from different plant species and tissues or growth conditions may show different electroporation tolerance. For instance, etiolated or greening maize mesophyll protoplasts tolerate electroporation extremely well.

Harvest protoplasts by centrifugation at 100x g for 1-2 min. Remove the supernatant. Freeze and store samples at -80°C until ready for analysis.

Add 100 µl of hypotonic buffer (10 mM Tris, pH 8 and 2 mM MgCl₂) or LUC lysis buffer (Promega), vortex vigorously for 2 sec to lyse the protoplasts. (The LUC lysis buffer contains 1% Triton X-100. Thus, gentle vortex is sufficient.)

A fast and economical xylenes extraction protocol is used for CAT assay (Seed and Sheen, 1988, *Gene* 67, 271-277; Sheen, in the supplement 9.6.5 of the *Current Protocols in Molecular*

Biology, Ausubel eds). Heating the cell extract at 65°C for 10 min in the presence of 5 mM EDTA might eliminate potential inhibitors for CAT assay (It seems to be useful for Columbia but not C24). We got good CAT activity without heat treatment. We use a Promega kit for LUC assay with a luminometer or a plate reader. The GUS assay has been described by Jefferson, R. (Add the cell extract into 10-100 μ l of 1 mM MUG, incubate for 30-90 min at 37°C, add 0.2-0.9 ml 0.2 M Na₂CO₃ to stop the reaction, and measure the fluorescence of MU).

C. Solutions

Enzyme solution

1-1.5 % cellulase R10 (RS is too strong) (0.1-0.15 g /10 ml)
0.2-0.4% macerozyme R10 (Yakult Honsha, Tokyo, Japan) (0.02-0.04 g/10 ml)
0.4 M mannitol (0.8M mannitol stock)
20 mM KCl (2M KCl stock)
20 mM MES, pH 5.7 (0.2 M stock)

Heat the enzyme solution at 55°C for 10 min (to inactivate proteases and enhance enzyme solubility) and cool it to room temperature before adding

10 mM CaCl₂ (1 M stock)
5 mM β -mercaptoethanol (optional)
0.1% BSA (Sigma A-6793 or A-7906) (optional) (10% stock, sterile)
The enzyme solution is light brown but clear (passed through a 0.45 μ m filter).

PEG solution (40%, v/v)

4 g PEG4000
(Fluka, #81240 can be purchased from Sigma since 2009) **VERY Important!!
3 ml H₂O
2.5 ml 0.8 M mannitol
1 ml 1M Ca(NO₃)₂ or CaCl₂ (no difference)

Washing and incubation solution (WI)

0.5 M mannitol,
4 mM MES, pH 5.7
20 mM KCl

W5 solution

154 mM NaCl (5M stock)
125 mM CaCl₂
5 mM KCl
2 mM MES (pH 5.7) (no glucose since we use glucose as a signal)

MMg solution

0.4 M mannitol
15 mM MgCl₂ (1M stock)
4 mM MES (pH 5.7)

D. Electroporation

40 ug plasmid DNA

4-6 x 10⁴ protoplasts/300 ul of 0.3 M mannitol/4 mM MES, pH 5.7/150 mM KCl

300-400 V/cm

5 msec, 200 uF, 1-2 pulses

E. Enzymes and nylon filters

There is no "catalogo number"

Here is what we ordered.

They call it "DESCRIPTION OF GOODS"

CELLULASE"ONOZUKA"R-10 (100g) (for Arabidopsis and dicot leaves)

CELLULASE"ONOZUKA"RS (100g) (for maize and monocot leaves)

MACEROZYME R-10 (100g) (for both dicot and monocot leaves)

The Cellulase and macerozyme are purchased from

Yakult Pharmaceutical IND. CO., LTD.

Shinbashi MCV Building

5-13-5 Shinbashi Minato-Ku

Tokyo, Japan

Tel 03-5470-8911 (international call 81-3-5470-8911)

Fax 03-5470-8921 (international fax 81-3-5470-8921)

The purchasing process can take up to a few weeks.

You can pay for express mail delivery, which takes 3-7 days.

You can send e-mail (yakultph@nifty.com) or call (81-3-5470-8911) to make an order request.

If you don't speak Japanese (I do not), please be very patient and insist on asking for someone who can speak English with you to make the order. Speak slowly and politely is the key!!

HIROTO WATANABE

DEPUTY GENERAL MANAGER

or

Haruhiko Kanai

Deputy Manager of Technical Department

(Note that the name of the manager may change over the years)

Yakult Pharmaceutical Ind. Co, LTD.

5-13-5, Shinbashi, Minato-Ku, Tokyo, Japan 105-0004

FAX : 81-3-5470-8921

Email : yakultph@nifty.com

URL: <http://www.yakult.co.jp/ypi/>

You may order the re-packaged Yakult enzymes from other companies (Google search), but they are in smaller package (10g) and much more expensive.

The Nylon filters (35-75 μm) can be purchased from Carolina Biological Supplies

F. Troubleshooting Tips

A list of factors that can be systematically tested if problems occur.

Arabidopsis accessions

Arabidopsis growth condition is accession-dependent (e.g., flowering time differences). Since leaves before bolting are used, Col and *Ler* plants will perform better under shorter photoperiod (10-12 h) and lower light.

Seed quality (age, growth condition)

Plates (B5 medium)

Soil

Growth condition (temperature, humidity, light intensity, photoperiod, water, nutrient)

Plants, Enzymes & DNA

Leaf age

Leaf development & morphology

Enzyme solution (enzyme quality, heat treatment)

Digestion time

Reporter genes

PEG

Mannitol

W5

Plasmid DNA quality (We use the more economical CsCl gradient for plasmid DNA purification. Make sure you remove the salt)

Protoplast/DNA ratio

Protoplast quality

Resting time

Response time

Incubation time (for gene expression or protein labeling)

Culture plates

Experiment size/duration (Very ambitious experiments tend to fail more often)

Stimuli application (timing, concentration, duration, etc.). We always test them extensively to cover a broad range of signal dosage or concentrations when a new signal is applied for the first time.

Arabidopsis plants are very sensitive to any kind of environmental changes. So, be sensitive to your plants' needs and behaviors.

