Naked DNA-transfer in protoplasts

Plant protoplasts can be obtained from a great variety of plant tissues, but leaves are the most effective source of protoplasts that will be used for transient expression during this course. Suspension cultures can also be used to prepare protoplasts, but for some unknown reason such protoplasts do not have the ability to synthesize much protein. In case of GUS, CAT, luciferase or any other sensitive enzyme assay used as reporter, this will not be a major problem, but when studying protein secretion and transport, and when Western blots are to be used as assay, it is important to use protoplasts with a high capacity to synthesize proteins. For this reason, the more labour intensive way of obtaining protoplasts from leaves is used. This can be done using the same protocol for plants such as lettuce, tomato, potato and *Arabidopsis* (the latter is very tedious due to the small leaves but it works). Leaves from *in vitro* grown plants usually digest faster and yield more protoplasts. For instance, when using *in vitro* grown tobacco plants, it is possible to produce 10^8 protoplasts on a day to day basis. Moreover, in vitro grown plants are sterile and the entire procedure can be kept sterile when working in a Laminar flow. Although experiments are fast and usually do not suffer from fungal infections, bacterial contaminations will spoil the experiment as they grow perfectly well in the sucrose containing mineral solution in which protoplasts feel happy. Bacterial infections usually arise from fingertips in the wrong place at the wrong time, as well as excessive talking and coughing nearby the open samples. During this practical, we will work at the bench and instruct participants to avoid bacterial infections.

BUFFERS

All solutions are sterilized by filtration through 0.2 μm filter in a laminar flow bench.

TEX Buffer

B5 salts	
MES	500 mg/L
$CaCl_2.2\ H_2O$	750 mg/L
NH_4NO_3	250 mg/L
Sucrose	0.4 M (13.7%)
pH 5.7 (with KOH)	

Digestion mix (10X) in TEX Buffer

2 % Macerozyme R10
4% Cellulase R10

The enzymes are mixed in the TEX buffer for 30 minutes then centrifuged (in 50ml Falcon tube) to remove insoluble particles prior to filter sterilization of the supernatant in a laminar flow bench. This solution can be stored in 50ml Falcon tubes in appropriate

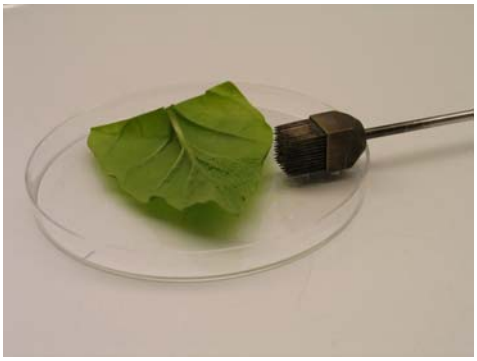
aliquots (5 ml) at -80°C, to be used directly for 10-fold dilution in TEX buffer and single use.

Electroporation buffer

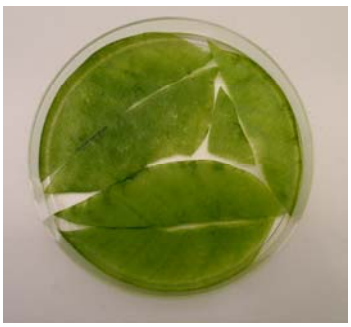
Sucrose	0.4 M (13.7%)
HEPES	2.4 g/L
KCl	6 g/L
$CaCl_2 2 H_2O$	600 mg/L
pH 7.2 (with KOH)	

PREPARATION OF PROTOPLASTS

1) Leaves are cut gently on the lower surface every 1-2 mm (without cutting through the whole surface). This is done by balancing the scalpel so that a fraction of its own weight exerts the pressure on the leaf surface. The leaf is held in place with a pair of forceps at the midnerve to minimize the damage at the leaf surface. It's also possible to punch holes on the lower surface by using a needle-bed which unfortunately is not commercially available but may be produced upon specific instruction by your local workshop.

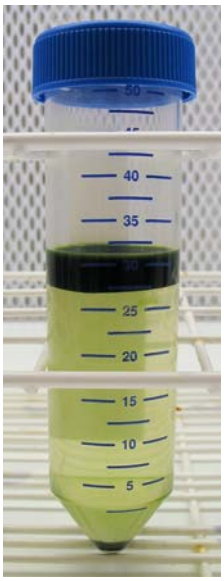


2) The midnerve is removed and the two halves of the leaf are transferred to a Petri dish containing 7 ml digestion mix (1X)., with the cut side facing downwards. It is possible to cut leaves and fill the surface of the liquid in approximately 5 minutes (with plenty of practice, as you will find).



3) The plates are incubated in the dark overnight, and 30 minutes before use, it is recommended to gently shake the plates to release protoplasts from the cuticula. Each plate should provide approximately $5x10^6$ protoplasts

4) The digestion mix is filtered through a 100 µm nylon filter and the filter can be washed with electroporation buffer. This will release further protoplasts from the tissue remnants and provide a first step in the adaptation to the new buffer. The protoplasts are then centrifuged in Falcon tubes (50 ml) for 15 mins at 100 g and room temperature in a swing-out rotor. Living protoplasts will move to the surface of the solution, where as cell debris will form a pellet or stay in solution.



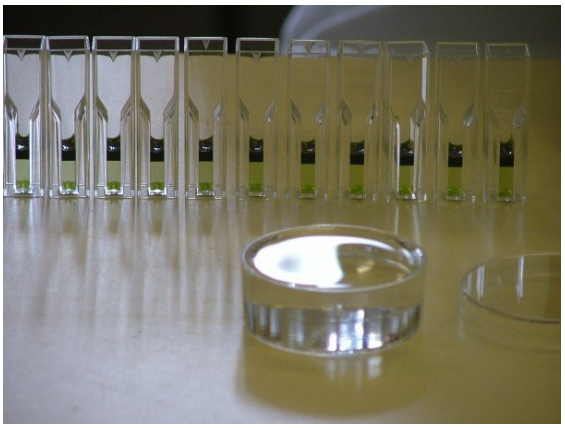
5) A long unplugged Pasteur pipette is connected to a peristaltic pump which can pump up to 1 litre per minute and the Pasteur pipette is inserted through the floating cell layer. To avoid that many protoplasts stick to the pipette and move down with it, a "window" is created by pushing the cells from the centre outwards. The pellet and the underlying medium are removed until the band of living protoplasts reaches the bottom. It is important to slow down the pump rate in advance. A programmable pump won't do for this, it is important to have a good old manually controlled pump.

6) Add 25 ml of electroporation buffer and spin again at 100 g for 10 mins. Remove the underlying solution as described above and repeat this procedure twice. The solution below the protoplasts should become clear and there should be hardly any pellet visible. If large quantities of protoplasts are produced (21 or more plates), a further washing step may be required.

7) At the end the protoplasts are resuspended in an appropriate volume in order to obtain 2-5 x10^6 protoplasts/ml. To determine the approximate number of protoplasts, there is no time to use a hemocytometre, and instead, the thickness of the floating protoplast layer is estimated. 7 plates of digested leaves should yield a layer of 5mm thickness under optimal conditions, which is sufficient for 15 electroporations and should be diluted to 8mL final. This solution is used for electroporation and can be stored for 1 hour. However, the sooner the cells are used, the better.

ELECTROPORATION PROCEDURE

1) 500 µl of protoplasts are pipetted gently into a disposable 1ml plastic cuvette using specially prepared blue tips of which the first 0.5 cm have been removed with a sterile scalpel. It is important to keep the suspension homogeneous by gently swirling the tube in regular intervals, otherwise the protoplasts will rapidly separate via floatation. It is also important to pipette the suspension gently to avoid shearing of the protoplasts when they are squeezed through the blue tip opening.



2) Plasmid DNA is diluted to 100 µl with electroporation buffer and mixed thoroughly prior to the experiment. This is important to allow efficient mixing of the DNA with the protoplasts. When pipetting plasmid mixtures for dose-response analysis, it is crucial to do so very accurately. Avoid pipetting less than 5 microliters and create appropriate dilutions of plasmids to obtain the desired range. If an effector is analysed, it is equally important to ensure that the reporter levels are identical in all samples. Add the 100 microliters to the 500 microliters of protoplast suspension and mix thoroughly by shaking (not stirring!! pipetting up and down is also not advised!!!). For optimal results, at least a quarter of the total volume of cell suspension should be occupied by cells. This can easily be verified after 5 minutes as the protoplasts will rapidly float.

3) The DNA/cell mixtures are incubated for 5 minutes (usually the time to finish pipetting the whole series) and can then be electroporated. We will use a home made electroporation device with a pair of stainless steel plate electrodes embedded in a teflon insulator, which will be directly inserted into the cuvette. No metal parts are exposed to the surface when the electroporation takes places. As this is a research instrument that is not for sale, it is not entirely fool proof, and it is i.e. possible to insert the electrode into the mouth and pull the trigger. This is not recommended. Commercial devices do not allow you to do this, but why would you? The electroporation is performed with the following conditions: 910 µF, 160 V. Please consult with the course organiser about ways to create home-built devices are advice regarding commercial appliances.

Between each electroporation, the electrodes are rinsed in distilled sterilized water to remove cell debris and DNA, dipped in 99 % ethanol to remove water, briefly passed through a flame to ignite and thus remove the ethanol, brief inspection of the electrode to ensure no liquid (=ethanol) is left, and then cooled down in electroporation buffer to remove any residual heat. This 4-step cycle allow swift progress without delay, but will mean that each electroporation takes approximately 1 minute.

4) The electroporated cells are incubated (without shaking) for 15-30 min to allow the cells to recover from the shock. During this period, holes in the plasma membrane are self-sealing, assisted by calcium ions present in the electroporation buffer. The cuvette is

then rinsed twice with 1 ml of TEX buffer and the obtained cell suspension is incubated in small Petri dishes in the dark during an appropriate time period (2 - 48 hours, usually 24 hours). Despite instructions from certain manufacturers of electroporation devices, note that if cells are resuspended in TEX buffer immediately after electroporation, no or hardly any transfected cells will survive, resulting in poor yield of recombinant proteins.

HARVESTING OF THE CELLS AND CULTURE MEDIUM

After incubation, the cell suspension is recovered in a small Falcon tube (15 ml). The further procedures depend on the particular application.

1) Intracellular proteins.

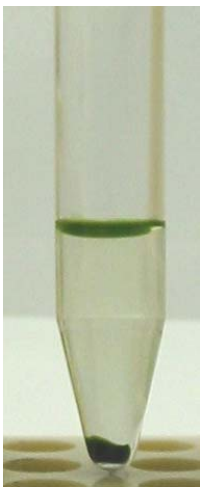
1mL of the cell suspension is diluted 10 fold with 250 mM NaCl and centrifuged for 3 minutes at 200 g. It is important to ensure that the bottom conical part of the tube has been mixed thoroughly prior to centrifugation, otherwise the protoplasts will sediment on top of a small remaining sucrose cushion that remains on the bottom, preventing the formation of a neat pellet. The supernatant can be completely removed with a peristaltic pump. The cell pellet is immediately placed on ice and can now be extracted with an appropriate buffer or frozen at -80°C (freezing cells in this manner has no influence on GUS activity, but we advise strongly against freezing at -20°C). Extraction of the fragile protoplast pellets is done by sonication, and unlike the manual grinding of leaves or other plant tissues, this method is highly reproducible, allowing a volumetric approach to biochemical experiments, thus removing the need for total protein quantification between samples of an experiment.

2) Secreted proteins

Secreted proteins are synthesized within the cell and transported to the extracellular medium. Therefore, these proteins will be present in the cells as well as outside the cells. Both fractions have to be analysed to see a relative distribution. Rates of secretion may vary depending on the protein of interest and the physioplogical status of the cells. Processing events may occur prior to secretion and co-expression of effectors or treatments with drugs may alter the distribution of the analysed protein. All this can be studied by analysing cells and medium of transfected protoplast suspensions.

To obtain the extracellular sample, a known amount of the cell suspension (i.e. 2 ml) is centrifuged at 100 g for 5 minutes, the living cells will float, the dead cells will pellet. A “window” can be opened in the floating protoplast layer using a sealed refined Pastuer

pipette. Then a portion of the underlying medium can then be removed manually with a refined Pasteur pipette (provided). The portion of medium is kept on ice for analysis (freezing of extracellular samples prior to processing is not recommended especially for analysis of α-amylase).



If the samples are to be analysed by SDS-PAGE it may be necessary to concentrate the medium. This can be achieved by any of the common methods, ammonium sulphate precipitation, chloroform methanol, TCA, etc…For enzyme assays the medium is usually just diluted with an equal volume of the appropriate extraction buffer (as in the case of α-amylase).

The intracellular sample is acquired from the remaining cell suspension after dilution (10 fold) with 250 mM NaCl, centrifugation and recovery of pellets as described in point 1).

3) Preparation of protoplast samples for microscopy

Protoplasts can be easily analysed by fluorescence microscopy with a little bit of improvisation. To avoid squashing them between the slide and the cover slip, we simply place standard electricians tape (100-150 microns thickness) with a small rectangular square cut out on the slide. Cells are first spun as in point 2, followed by the removal of the pellet of dead cells and most of the underlying medium, so that only floating cells remain in a small volume (0.5 mL). This ensures that the cell density is high enough for rapid screening and identification of transfected (=fluorescent) cells. 50 microlitres of cells (using a cut tip to minimise shearing) is pipetted into the cut-out rectangle, after which the cover slip is carefully placed on top. Protoplasts will have enough space in the rectangle to move freely, but will float up and settle close to the glass surface of the cover slip. This will stabilise the protoplast, which can then be imaged directly under the microscope.