General protocols

Preparation of electroporation competent 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. 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. However, when using in vitro grown tobacco plants, it is possible to produce $10^8$ protoplasts on a day to day basis. The following protocol has been used successfully with leaves from other plants such as lettuce, tomato, potato and Arabidopsis (the latter is very tedious due to the small leaves).

BUFFERS

TEX Buffer

- B5 salts
- 500 mg/l MES
- 750 mg/l CaCl$_2$ (2 H$_2$O)
- 250 mg/l NH$_4$NO$_3$
- 0.4 M sucrose (13.7%)
- pH 5.7 (with KOH)

Digestion mix (10X) in TEX Buffer

- 2 % Macerozyme R10
- 4% Cellulase R10
- 30 minutes resuspension, centrifugation (in 50ml Falcon tube) to remove unsoluble particles and 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

- 0.4 M Sucrose (13.7%)
- 2.4 g/l HEPES
- 6 g/l KCl
- 600 mg/l CaCl$_2$
- pH 7.2 (with KOH)
All solutions are sterilized by filtration through 0.2 µm filter with a syringe in a laminar flow bench.

**PREPARATION OF PROTOPLASTS**

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 (ask for demonstration prior to starting).

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). Each plate should provide 5x10⁶ protoplasts.

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.

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 80 g and room temperature in a swing-out rotor. Living protoplasts will move to the surface of the solution, while the cell debris will form a pellet or stay in solution.

A long 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 (requires demonstration). 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 this, it is important to have a good old manually controlled pump, the left hand controls the pump, the right hand holds the pipette.

Add 25 ml of electroporation buffer and spin again at 80 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.

At the end the protoplasts are resuspended in an appropriate volume in order to obtain 2-5 x10⁶ protoplasts/ml. This solution is used for electroporation and can be stored for 1 hour. The sooner the cells are used, the better.
ELECTROPORATION PROCEDURE

500 µl of protoplasts are pipetted gently into a disposable 1ml plastic cuvette. It is important to do this gently to avoid shearing of the protoplasts when they are squeezed through the blue tip opening. 10 to 50 µg of DNA in 100 µl of electroporation buffer are added and mixed by gentle shaking.

The cells 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, 130 V.

To remove cell debris and DNA, the electrodes are rinsed in distilled sterilized water, dipped in 99 % ethanol, briefly flamed (the ethanol and flaming are just to dry the plates) and cooled down in electroporation buffer to allow swift progress without delay.

The electroporated cells are incubated (without shaking) for 15-30 min. Living cells will have floated to the surface and it is possible to remove 300 - 400 µl of underlying electroporation buffer and cell debris. 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).

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 reporter genes such as GUS and luciferase

The cell suspension is centrifuged at 80 g for 5 minutes to float the cells. The underlying solution is removed with a fine Pasteur pipette and the remaining suspension is diluted 10 fold with 250 mM NaCl. A further 5 minutes centrifugation will result in a cell pellet and 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 in cells has no influence on GUS or luciferase activity).

2) Secreted proteins such as α-amylase
Remove 500 µl of the cell suspension, and place on ice in an Eppendorf tube. The suspension can be sonicated later and centrifuged to obtain the **total sample**. This can be used later to find out if anything has been lost in the following procedures.

Centrifuge a known amount of the same cell suspension (i.e. 2 ml) at 80 g for 5 minutes, the cells float and the underlying medium is removed gently with fine pipette (**extracellular sample**) and kept on ice.

The remaining cell suspension is diluted with 250 mM NaCl, centrifuged as described under 1 and the pellets are placed on ice. Extraction of these cells will provide the **intracellular sample**, and it is possible to calculate the concentration factor when a known amount of extraction buffer is used (the cells originate from i.e. 2ml suspension).

The total- and extracellular sample are undiluted compared to the cell suspension, but the intracellular sample may be concentrated. It is important, however, to be able to calculate the concentration factor compared to the original cell suspension.

### 3) Special cases

Sometimes, it is important to determine if the transgene has been transcribed (i.e. if the protein could not be detected). In this case one should use short incubation times after electroporation, as the mRNA levels will reach a plateau soon after electroporation due to the low mRNA stability. Prolonged incubations will result in lower mRNA levels as the plasmid is slowly degraded in the cells. This is also true for in vivo labelling reactions, as the protein synthesis rate will depend on the mRNA level. For in vivo labelling, protoplasts are floated as described above and concentrated to allow a minimal use of radioactively marked aminoacids (usually a mixture of methionine and cysteine). Labelling is usually between 30 minutes and 3 hours. Labelled cells can then be washed with 250mM NaCl to remove free label and obtain a small pellet which can be extracted. In vivo labelling is used to measure the half-life of a protein or to detect small peptides which cannot be blotted into membranes and detected by Western blots.