

E X E R C I S E

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Transient Transfection of Mammalian Cells

This basic transfection procedure is applicable to most adherent cell types. Certain cell types that do not display robust pinocytosis or membrane turnover may be more difficult to transfect. As with all techniques, fine-tuning for optimal conditions for a particular cell type comes with practice and experience with that cell type. The volumes listed are appropriate for 33 mm² plates and would be increased or decreased for other plate sizes.

We will introduce a *plasmid* into the cell via liposome fusion; specifically using Lipofectamine, a commercial reagent. Many different such transfection reagents exist, and the optimal one for each cell type can vary. We achieve $\geq 90\%$ successful transfection of NB2a/d1 cells using Lipofectamine, yet only approximately 5% with certain newer reagents that are demonstrably superior to Lipofectamine in other cell types.

The plasmid will be the pSV-beta-galactoside vector. This vector, when successfully transfected and expressed, will cause the cells to produce b-galactoside, which is a protein that we can then assay. Researchers often insert a gene-of-interest in this vector by recombinant methods well-described in the manufacturer's instructions. This particular vector is often utilized in co-transfection studies to provide evidence that another vector (in most cases, a vector lacking an easily-assayed product) has been introduced to the cell. A blue/green colored product, visible under bright field microscopy, will be produced by transfected cells; this vector, therefore, allows the use of standard microscopes for confirmation of transfection. Many vectors with a fluorescent reporter are commercially available, but this requires a UV microscope.

** You will be provided with two 35 mm² stock plates per group.

PROTOCOL

Part I: Formation of DNA/Liposome Complexes:

****NOTE** THIS MUST BE DONE INSIDE OF THE CULTURE HOOD TO MAINTAIN STERILITY!!**

- Mix 0.5 μ l of the vector solution with 100 μ l of serum-free media.
- In a separate, sterile microcentrifuge tube, mix 8 μ l of Lipofectamine solution with 100 μ l of serum-free media.
- Combine the contents of both tubes (i.e., the Lipofectamine and vector tubes) into a single tube, mix gently, and incubate at room temperature for 45 minutes. This is to allow for the formation of DNA-liposome complexes. This solution may become cloudy.

**Because of the small quantities of materials used in this experiment, you will be provided with the vector solution and Lipofectamine solution in separate tubes.

- As the incubation time draws close: Add 800 μ l of serum-free media to the DNA-liposome solution (total volume is now approximately 1 mL); mix gently.
- Then rinse each plate twice with 2 mL of serum-free media.
- Add 1 mL of the transfection solution to the plate gently.
- Incubate for 3.5 hours at 37°C.
- Then WITHOUT REMOVING THE TRANSFECTION MIXTURE, add 1 mL of 10% serum-media.
- Incubate for 24 hours at 37°C, then change the media, i.e., remove the transfection solution and add 2 mL of 10% serum-media (DO NOT RINSE WITH SERUM-FREE MEDIA).
- Then incubate for 72 hours at 37°C.
- This incubation gives the cells time to express their new DNA and accumulate some protein product.

Part II: Cell Fixation and Product Assay:

Both solutions will be provided.

- Fixation:
This can and should be performed outside of the hood.
- Remove 1 mL of the media from the plate.
- Then add 1 mL of 2% paraformaldehyde to the plate and incubate for 10 minutes at room temp.
- Rinse each plate with 1 mL of PBS, and then add 2 mL of PBS.
- Plates can then be wrapped in parafilm and stored in the refrigerator.
- Assay:
The provided staining solution consists of:
5 mM potassium ferricyanide
5 mM potassium ferrocyanide
2 mM MgCl₂
1mg/mL of X-gal (the substrate, which will yield a blue precipitate upon hydrolysis)
- After adding the staining solution, the plates should then be examined under phase optics on the microscope. If the staining is very faint, examination under bright field may help. Score the efficiency of successful expression by determining the percent of cells with blue stain versus those without; a “ball-park” score is fine.

An important control is to include a non-transfected plate in this staining procedure, to confirm that the staining is associated with the vector, not the native cells. You could also include a mock-transfected plate (i.e., carry out the entire procedure above, including Lipofectamine, but do not mix in the vector).

Note that, when you observe beta-galactosidase activity, it indicates that you have successfully coaxed your cells into taking up the construct, retaining it, expressing it, and having a function enzyme generated. This is a powerful procedure that becomes more and more a standard feature of cell culture each day.

Transient versus Stable Transfection:

When cells are transfected and analyzed, as above, a proper way to refer to this is transient transfection, since we have no indication whether the cells will retain the plasmid. A minority of cells may incorporate the plasmid into their chromosomal DNA, which renders them stably-transfected.

The vector used herein, and most commercial vectors, contain the sequence for neomycin resistance. As a corollary of this exercise, one could add gentamycin or another appropriate antibiotic to the medium of a plate transfected as above; peruse recent literature articles and the instructions provided with the plasmid for the appropriate antibiotic(s) and concentration(s). Only those cells that contain and continue to express the vector will survive initially, and when cells divide, the vector is not passed on to both daughter cells. Only those cells that integrate the vector into their chromosomal DNA (stably-transfected) will survive for any length of time. Accordingly, >99% of the culture will likely die out. However, maintaining what seems to be an empty culture dish for a week or two, after changing the medium to remove all the dead cells, may yield a few colonies of surviving cells. These can be carefully removed with a micropipette and placed in 96-well trays. Subclonings should be carried out to ensure that you don't have a mixture of two such colonies (dilute until you observe only 1 cell per well...use conditioned medium...). Once you have established a line or two, test some plates of these cells for expression of beta-galactosidase. This additional exercise can be carried out "in the background" while conducting other exercises.

MATERIALS

pSV-beta-galactoside vector
Lipofectamine
Sterile micro-centrifuge tubes (2 mL tubes)
Serum-free media
10% FBS-containing media
2% paraformaldehyde
Staining solution
1X PBS

- pSV-beta-galactoside vector:
 - Make 0.5 mL aliquots into sterile tubes.
- Lipofectamine:
 - Make 8 mL aliquots into sterile tubes.
- Serum-free media:
 - Refer to Introductory Lab.
- 10% FBS-containing media:
 - Refer to Introductory Lab.
- 2% paraformaldehyde solution:
 - Using 16% stock solution, dilute to 2% using dH₂O.
- To make 40 mL of 2% solution:
 - Dissolve 5 mL of 16% stock solution into 35 mL of dH₂O.

- Staining solution:
 - 5 mM K Ferricyanide
 - 5 mM K Ferrocyanide
 - 2 mM MgCl₂
 - 1 mg/mL of X-gal
- To make 50 mL of the staining solution:

Dissolve 42.24 mg of potassium ferrocyanide, 82.31 mg of potassium ferricyanide, 9.52 mg of magnesium chloride in 50 mL of PBS.
X-gal is added as 1 mg/mL.

HINTS

- The staining solution should be made relatively fresh and stored in amber-colored bottles.
- The vector solution should be incubated with the serum-free media for at least 5 minutes. This will allow for better transfection rates.
- Color development can take approx. 20-30 minutes. Toggling between phase and bright field microscopy will yield better results.