CHAPTER 14

TITLE

Lipid-mediated transient tranfection in A549 cell line

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Summary

Trials of transfection in eukaryotic cells are essential tools for the study ofgene and protein function. They have been used in a wide range of research fields. In this chapter a method of transient transfection of the A549 cell line, human lung cells of alveolar epithelium, with an expression plasmid is described. In addition, the fundamental characteristics of this experimental procedure are addressed.

Keywords: transfection, expression vector, lipofection, lipofectamine.

1 INTRODUCTION

1.1 Cellular transfection

In the field of molecular and cellular genetics, we use the term transfection to refer to the process whereby exogenous genetic material is introduced into cultured eukaryotic cells using non-viral mechanisms. The development of these techniques, taking as a base model the introduction of DNA in *E. coli* (bacterial transformation), has driven notoriously the advancement in the understanding of the processes of gene regulation and the mechanisms of protein expression and function in cellular systems (***1***).Transfection techniques allow performing protein expression in a eukaryotic system in which they will carry out post-translational modifications necessary for correct operation (absent in prokaryotes models) and therefore, more accurate studies of cellular activity.

Two types of transfection can be distinguish depending onwhether the DNA molecule is integrated into the genome of the host cell, referring as transient or stable transfection respectively (***2***). Transient transfections provide a high degree of gene expression and allow co-express several proteins at the same time. But, only temporary protein expression with high variability in the percentage of expressing cells will be obtained.

Conversely, when a stable transfection is carried out, the resulting cell population will be a homogeneous clonal population with theoretically continuous production of protein. In this case, the level of expression will be moderate and it will be difficult to simultaneously co-express multiple proteins. In addition, the selection of the clones that have integrated the plasmid is tedious and requiresthe incorporation of a plasmid containing a gene for antibiotic resistance to select and perpetuate the clones (***3***).

1.2 Transfection methods

For an efficient transfection, different barriers need to be overcome, such as the entry of genetic material into the cell, their release of the endosome and escape of lysosomal degradation, and finally, its translocation to the nucleus. Several transfection methods have been developed in recent decadesto solve these problems (***4***).

To surmount the first barrier the electrostatic opposing forces have to be neutralized because the DNA molecule and cell membranes are both negatively charged. To this end, numerous methods have been developed. Generally,transfection assays can be divided into chemical methods seeking to coat the DNA molecules with positive charges, and physical methods, whereby pores are created in cell membrane or the DNA molecule is mechanically introducedinto the cell (***5-11***).

An ideal transfection method would meet the following criteria: firstly, it should be easy to perform, with minimum costs and efforts; secondly, the method should be suitable for both stable and transient transfection; and finally the procedure should be highly versatile, i.e., it should be valid for any cell type (***12***)(*see***Table 1**).

1.3 Expression vectors

The progress in the understanding of mammalian genes has been largely due to the development and use of expression vectors in cell culture. Many proteins are expressed at low levels in cells culture therefore, expression vectors that increase protein synthesis by strong promoters are usedto their study. In addition, the expression vector systems allow characterizing the impact of specific mutations on cell metabolism and their ability to stably alter the cellular phenotype as a function of transgene expression (***14***).

Depending on the final purpose of the study, the expression system that best fits the needs should be chosen. There are vectors that amplify the synthesis of fusion proteins using a strong promoter along with the genes of interest. Other systems allow studdingthe mechanisms of gene expression regulation by combining promoters and transcription factors to the gene of interest, or to analyze the effects caused by certain treatments or conditions on the protein expression.

The expression vectors should have certain common features that facilitate their use in this type of assay and contribute to efficiently express the exogenous gene within the target cell (***15***). Although these characteristics may vary depending on the study peculiarities, these features overall can be summarized as follows:

1. A prokaryote autonomous replication origin that permits plasmid amplification in bacterial systems.

2. A eukaryotic replication origin that makes possible the plasmid expression in the target cells.

3. A promoter of the gene of interest recognized by a mammalian polymerase. The signal sequences of the transcription start are essential for the expression of foreign protein. Most common promoters are viral, although other bacteriophage promoters such as constitutive or tissue specific, or even inducible promoters can also be used.

4. A multiple cloning region where the cDNA encoding the protein will be inserted.

5. A transcription terminator.

6. A ribosome-binding site.

7. Antibiotic resistance genes that allow a first selection of the bacterial clone with the plasmid, when the vector amplification is performed in a bacterial system. The most widely used is the resistance to ampicillin. Other selection genes can be incorporated when a stable transfection is carried out, for example neomycin resistance gene or dihydrofolatereductase, which confers resistance to methotrexate.
8. Regulatory gene sequences.
9. Fragment of DNA for homologous recombination in order to achieve stable transfections.

The characteristics of an ideal vector for our purpose should meet the following requirements (***16***):

1. It has to be reproducible.
2. It has to be stable.
3. It has to permit the insertion of genetic material without size restriction.
4. It has to reach high concentrations.
5. It has to enable specific integration of gene.
6. It has to discriminateand act on specific cells.
7. The gene expression should be under control/regulation.
8. It has to be fully characterized.
9. It has to be innocuous and with negligible or no side effects.
10. It has to be easy to produce and store at a reasonable cost.

The huge number of published papers that employ this technology corroborates the wide range of applications resulting from the use of expression vectors. Some of the most remarkable applications are:

1. The study of recombinant proteins.
2. Therapeutic applications of modified cells.
3. Studies of protein expression and protein function.
4. Studies of gene regulation (promoters, regulatory elements...) (***17***).
5. Generation of transgenic organisms.
6. Gene Therapy.
7. The expression of protein for purification.
8. The study of RNA processing.
9. The study of protein interaction.
10. Subcellular localization of proteins.

In this chapter a method of transient transfection of the A549 cell line, human lung cells of alveolar epithelium, will be described.

2. MATERIAL

2.1 Instrumentation and consumables

1. 24-well cell culture cluster (other culture plates can be used)
2. Laminar flow cabinet
3. CO_2 incubator

4. Thermostatic bath
5. Hemocytometer
6. 1.5 ml microcentrifugue tubes
7. 15 ml sterile tubes
8. 50 ml sterile tubes
9. Automatic pipettes and appropriate tips
10. Electric pipettor
11. Inverted phase contrast microscopy
12. Centrifuge
13. Microcentrifuge
14. NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific)
15. Commercial purification kits plasmid DNA (i.e.,Qiagen Plasmid Maxi kit)

2.2 Culture Solutions

1. Trypan Blue Solution 0.2 μm filtered
2. Trypsin-EDTA solution (1X)
3. Ethanol 70%
4. Fetal Bovine Serum (FBS) sterile
5. RPMI 1640 medium with L-Glutamine (or other appropriate medium) RPMI 1640 medium with L-Glutamine supplemented with antibiotics and fetal bovine serum 10%.

6. Antibiotics: Penicillin (100 U/mL) / Streptomycin (100 μg/mL). Anti-microbial combination.
7. PBS 1X: 1.37mMNaCl, 2.6mMKCl, 10mM Na2HPO4, 1.8mM KH2PO4. Adjust the pH to 7.4 with HCl.

8. H_2O sterile.

2.3 Cell culture and DNA plasmid

1. Human alveolar basal epithelial cells, A549. They can be purchased in American Type Culture Collection (ATCC) website (http:/www.atcc.org).
2. pCMV6-Entry plasmid:Expression vector with strong constitutive CMV promoter.
3. pCMV6-PTGDR plasmid: pCMV6-Entry vector in which the gene encoding the prostaglandin D2 receptor (*PTGDR*) has been cloned in the polylinker.
4. pUC18 plasmid: Vector used as DNAcarrier, to stabilize the total amount of DNA transfected in all experimental conditions.

2.4Transfection reagents

1. Opti-MEM® I Reduced Serum Medium (Gibco, Life Technologies, Thermo Fisher Scientific)
2. Lipofectamine® 2000 Reagent (Invitrogen, Life Technologies, Thermo Fisher Scientific)

3. METHODS

The lipofection methods are not universal, and vary widely among different cell lines. Due to the wide variety of factors that determine the efficiency of transfection methods, the optimal conditions should be identifiedfor each cell type and for each type of test. Before any testing with cell lines, we recommend consulting the American Type Culture Collection (ATCC) website (http://www.atcc.org) for helpful information about optimal growth media and specific serum.

3.1 Factors influencing transfection efficiency

There are several important factors to be consideredto achieve a successful Lipofectamine-mediated transfection experiment (***18***):

1. Serum in cell culture prior to transfection:Fetal bovine serum contains heterogeneous concentrations of hormones, proteins and other biomolecules that may interfere in subsequent experimental results. Firstly, we should evaluate the possibility of eliminating or reducing the concentration of fetal bovine serum from10% to 2-3%, at the time of seeding cells for expression assays.

   Notice that the reduction of serum in the culture medium slows cell growth and may reduce the cellviability causing low transfection efficiencies.

   An alternative to serum starvation is the use of a modified serum with low concentrations of some of its components.

2. Presence of serum in transfection:Although there are currently commercial reagents that are not interfered by fetal bovine serum, the presence of serum in the culture medium can inhibit the reaction of transfection due to interference in the formation of the DNA-lipid complexes for some of its proteins. In such cases, it is recommended to dilute DNA to transfection and dilute the reagents of transfection in specific medium with low serum concentration such as Opti-MEM® I Reduced Serum Medium.

3. Antibiotics in culture medium: Under general conditions the medium for cell culture is supplemented with antibiotics to prevent contaminations. However, lipofectamine, or cationic lipids in general, cause an increase in the permeability of cellular membranes. As a result, the antibiotic penetrates into the cells in

greater quantities causing cytotoxicity and deterioration of the transfection efficiency (***19***).

4. Cell density: It is recommended to maintain the cell culture at a density of 60-80% of confluence to ensure high efficiency in the transfection process (*See***Figure 1)**.
5. Cell viability: Cells must be in exponential growth phase at the time of transfection. In addition, it is advisable not to use cells that are in or above 30 passages after thawing the vial of a stock culture, to prevent changes in the characteristics of the cell culture as a result of repeated passages (***19***).
6. Type of plasmid DNA vector: The promoter coupled to the expressed gene must be compatible with the cellular system chosen. The size and the topology (linear or supercoiled) of the plasmid DNA vector affect the transfection efficiency.
7. Amount of DNA: The optimal amount of DNA used in transfection assays need to be optimized depending on cell type, experimental conditions and the procedure used.
8. Quality of DNA: For efficient transfection, the transfected DNA molecule must be free of protein, RNA and chemical contaminants such as formaldehyde, isopropanol, or ethanol used in the purification process.

3.2 Premises:

1. The whole process should be done in laminar flow cabinets.
2. Decontaminate all surfaces with 70% ethanol.
3. Use sterile material.
4. Discard any material or culture suspected of being contaminated.

3.3 Preparation of expression vectors

Before starting the transfectionprocess, prepare the vectors at the appropriate concentration of use. In this experiment the vectors used are pCMV6-Empty, pCMV6-PTGDR and pUC18 (*See***Figure2**).

Plasmids are amplified by bacterial transformation, purified using commercial purification kits plasmid DNA andeluted in H_2O.

Plasmid DNA concentration is quantified spectrophotometrically. Verified that the 260/280 nm absorbance ratio, which indicates the purity of the extracted DNA, is around 1.8 accepting then the plasmid DNA is of good quality. Furthermore, the 260/230 ratio must be between 2.0-2.2 considering that ratios below this value show a phenolic solutions contamination that may alter subsequent experimental procedures (**20**).

Dilutions should be maid to obtain a working solution DNA.

Plasmid solutions are aliquoted and stored at -20 until use.

3.4 Protocol (*See* Figure3)

3.4.1 Day 1: Seed 50,000 A549 cells in medium without antibiotics in 24-well plates. (See Note 1).

1. Remove and discard the culture medium.
2. Wash with PBS to remove all traces of serum that contains trypsin inhibitor.
3. Detach adherent cells by trypsinization.
4. Neutralize the trypsin with pre-warmed complete RPMI medium by incubating in a 37ºC water bath. (*See* **Note 2**)

5. Recount the cells by vital staining with Trypan Blue solution, counting in Neubauer chamber or similar.
6. Resuspend the cells in a volume of pre-warmed medium to obtain a density of 1×10^6 cells/ml.
7. Seed 50,000 cells per well in a total volume of 500µl of RPMI medium supplemented with 10% fetal bovine serum <u>without antibiotics</u>. (*See* **Notes 3, 4 and 5**).
8. Ensure uniform distribution of the cells in the wells and prevent cell clumps. (*See* **Note 6**).
9. Maintain 20-24 hours in incubation at 37ºC and 5 % CO_2. The cell density should be between 60% and 80% at the time of performing the transfection process. (*See* **Notes7 and 8)**.

It is required to make appropriates controls in each transfection assay. Include a negative control without DNA to check optimal cell growth conditions. To determine problems related with the insert, transfect the cellswith a plasmid without the gene of interest (i.e. include only the plasmid backbone used to construct the expression vector). Including a positive control is also advisable.

3.4.2 Day 2: Transfection

1. <u>Making the mixture **A**: DNA + OptiMEM.</u>

   Dilute the plasmid DNA in Opti-MEM® I Reduced Serum Medium in the corresponding pre-labelled Eppendorf tubes.

According to the manufacturer`s instructions transfect cells with 500 ng of final DNA per well, in a final volume of 50 µl ofOpti-MEM®I ReducedSerum Medium per well. Perform each reaction mix in triplicate.

(*See* **Notes 9, 10 and 11)**(*See***Table 2)**

Incubate at least 5 minutes at room temperature.

2. <u>Making the mixture **B**: Lipofectamine + Opti-MEM</u>.

   Prepare 1 µl of lipofectamine and 49 µl of Opti-MEM®I Reduced SerumMedium per well. Mix by pipetting up and down several times (do not vortex). (*See* **Note 12**)

   Lipofectamine: 1ul/well x 3.5 (triplicate) x nº tubes mixture A

   Opti-MEM: 49ul/well x 3.5 (triplicate) x nº tubes mixture A

   Incubate 5 minutes at room temperature and protected from light.

3. <u>Making the transfection mixture (A+B)</u>.

   Add 175µl of mixture **B** (lipofectamine+Opti-MEM) to each tube of 1.5 ml containing mixture **A** (DNA+Opti-MEM), (Ratio 1:1). (*See* **Note 13**)

   Incubate 20-30 minutes at room temperature to allow formation of the DNA-liposome complex. Protect from light. (*See* **Note 14)**

4. <u>Incubation of the cells with the transfection mixture.</u>

Use cells seeded the day before. Remove the culture medium and perform washing with PBS carefully not to disturb the cells. (*See* **Notes 15 and 16)**

Add to each well 100 µl of the transfection mixture **A** + **B**(DNA+lipofectamine+ Opti-MEM®I Reduced Serum Medium).

Add to each well 100 µl of de Opti-MEM®I Reduced Serum Medium to ensure that the cells are completely covered with medium.

Maintain the cells in the CO_2 incubator with the transfection mixture for 4-6 hours.

After the incubation period, remove transfection mixture, with care not to lift the cells, and add 200µl complete RPMI medium with antibiotics. Optional: perform washes with PBS between media change.

Maintain 12-72 hours in incubation at 37ºC and 5% CO_2. (*See* **Note 17)**

5. <u>Harvesting cells to analyze transfection.</u>

The collection of the cellular extracts, which are needed according to the designed experiments (DNA, RNA, proteins, cytokines...), can be performed from 12 hours after transfection up to 72 hours (***14***), depending on cell type and the activity of the promoter integrated the vector.

4. Notes

1. The volumes of the transfection reaction are scalable depending on the tissue culture format. It is recommended to perform triplicates for each condition of the experiment in order to ensure the reproducibility of the assay.
2. After tempering or heating any vial in a water bath, wash the surface of the container with ethanol 70% before introducing it in the laminar flow cabinets, in order to avoid possible contamination.
3. Serum can reduce the efficiency of transfection. Notice that the absence of serum can make the liposome more toxic for cells.
4. The liposome complex can interact with the antibiotic decreasing the effectiveness and increasing the toxicity.
5. Change cell numbers proportionally for different size plates.
6. For optimal results it is important to have a single cell suspension. It is advisable to pipette up and down when seeding the cells, and gently rock the plate to ensure good distribution.
7. Adjust the number of seeded cells and transfect when cells are 60% -80% confluent.
8. In order to stabilize and recover the culture from trypsinization it is recommended to carry out the cell seeding at least 24 hours prior to transfection assay.
9. Use an innocuous plasmid vector to complete up to 500ng per well when it is not possible to use the target vector (i.e. pUC18).
10. Follow the manufacturer's specifications for the amount of DNA to transfect.
11. Check the accurate quantity of expression vector to transfect. (*See***Table 2**).

12. It is recommended to perform a whole reaction mix for triplicates taking into account pipetting variations, in order to save time and reactives(i.e. multiply the single reaction volume by 3.5 to get enough transfection mix for 3 replicates wells including an extra for pipetting error).
13. The DNA: lipofectamine ratio can be varied from 1: 0.5 to 1: 5 to adjust the efficiency.
14. Incubations lasting more than 30 minutes may result in a decrease in transfection efficiency.
15. Ensure that the cells are not so long uncovered with no solution to prevent drying and suffering. Accordingly the exchange of the culture medium, the washing, and the change of transfection medium must be quickly performed.
16. When washing, deposit the PBS on the wall, by gently pipetting, not directly on the cells.
17. The time of incubation with the transfection mixture can be varied depending on the cell type used.

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Table 1.Characteristics of different transfection methods.

Method		Foundation	Comments	
			Advantages	Disadvantages
Chemicals	Calciumphosphate	Precipitation of the DNA molecules calcium phosphate forming an insoluble complex which is introduced into the cell by endocytosis.	- Simple Method - Stable and transient transfection	- Low reproducibility - Variable efficiency - Only in vitro techniques
	DAE-dextran	Cationic polymer that binds to DNA and introduced into the cell by endocytosis	- Simple Method - High efficiency - Reproducibility - Low Cost	- Transient transfection - Toxicity - Only in vitro techniques
	Lipofection	Formation of colloidal particles with lipid membranes surrounding and DNA molecules are introduced into the cell by endocytosis or membrane fusion	- High efficiency - Applications in vitro and in vivo - Not immunogenic - High versatility. - User friendly - Can carry DNA molecules larger sizes	- High cost - More effective in adherent cells for cells in suspension

Physical	Electroporation	Open pores in the plasma membrane by electric pulse of high intensity and short duration.	- Simple technique - High reproducibility - High efficiency - Transient and stable transfection	- High cell death - Requires cell resuspension - High cost
	Microinjection	Introduction through fine glass capillaries of solutions of macromolecules under control by microscopy	- High efficiency - Highlyversatility	- Complex technique -Optimization of multiple parameters - Handling of individual cells (high time consumption) - High cost

	Biolistic	Microparticle bombardment. Genetic material binding to biologically inert particles (tungsten or gold) and the cell membrane penetration at high speeds	- Useful in cells resistant to other methods - Useful in immunization trials (***13***) - High reproducibility - Low amount of DNA - Limited cell manipulation	- Lower efficiency electroporation or lipofection - Preparation of microparticles - High cost - Requires specific instrumentation

Table 2.Example of a transfection assay.

volumes (µl)	single cells	25 ng empty vector	25 ng expression vector	50 ng empty vector	50 ng expression vector	100 ng empty vector	100 ng expression vector
pCMV6-PTGDR **(25ng/µl)**	--	--	1	--	2	--	4
pCMV6 empty **(25ng/µl)**	--	1	--	2	--	4	--
pUC18(carrier) [**250ng/µl**] up to 500ng	2	1.9	1.9	1.8	1.8	1.6	1.6
optimem (up to **50 µl**)	48	47.1	47.1	46,2	46,2	44,4	44,4

Figure 1.

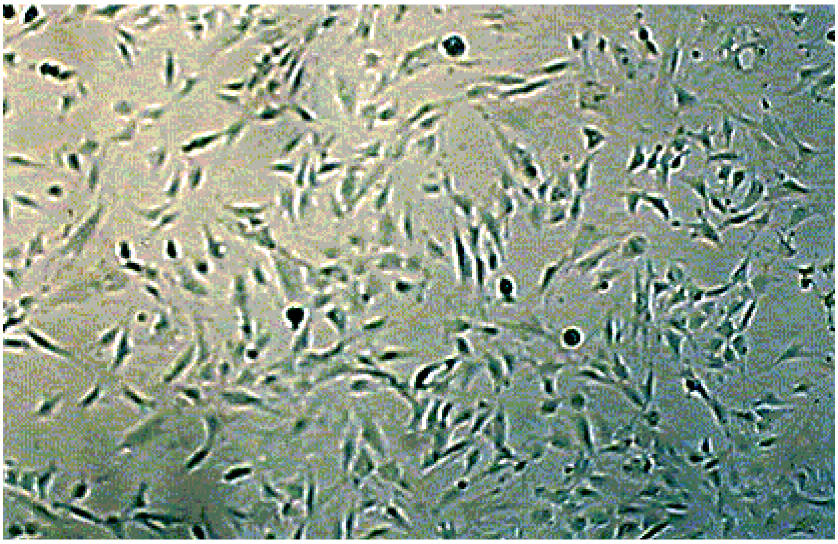


Figure 2.

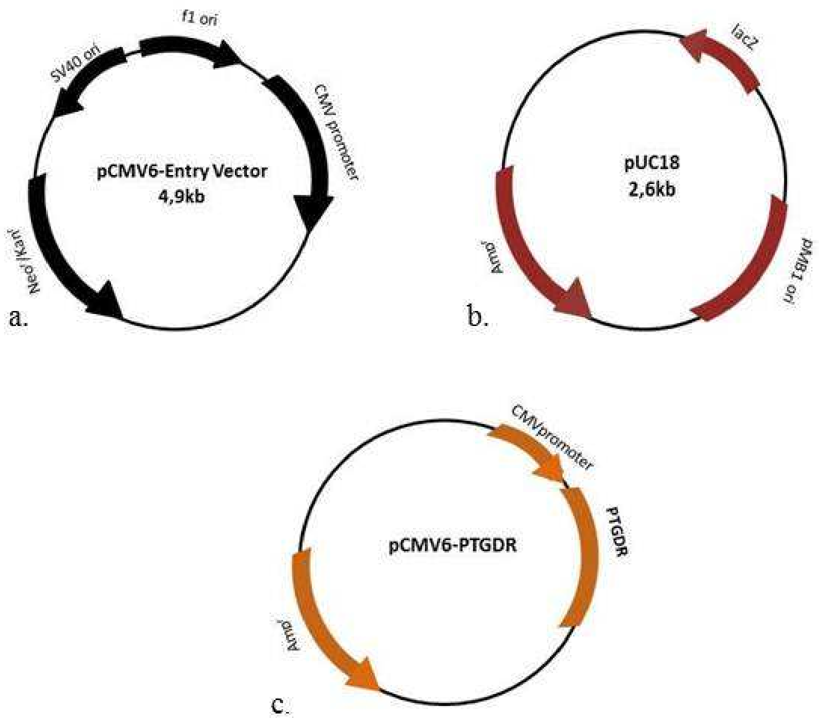
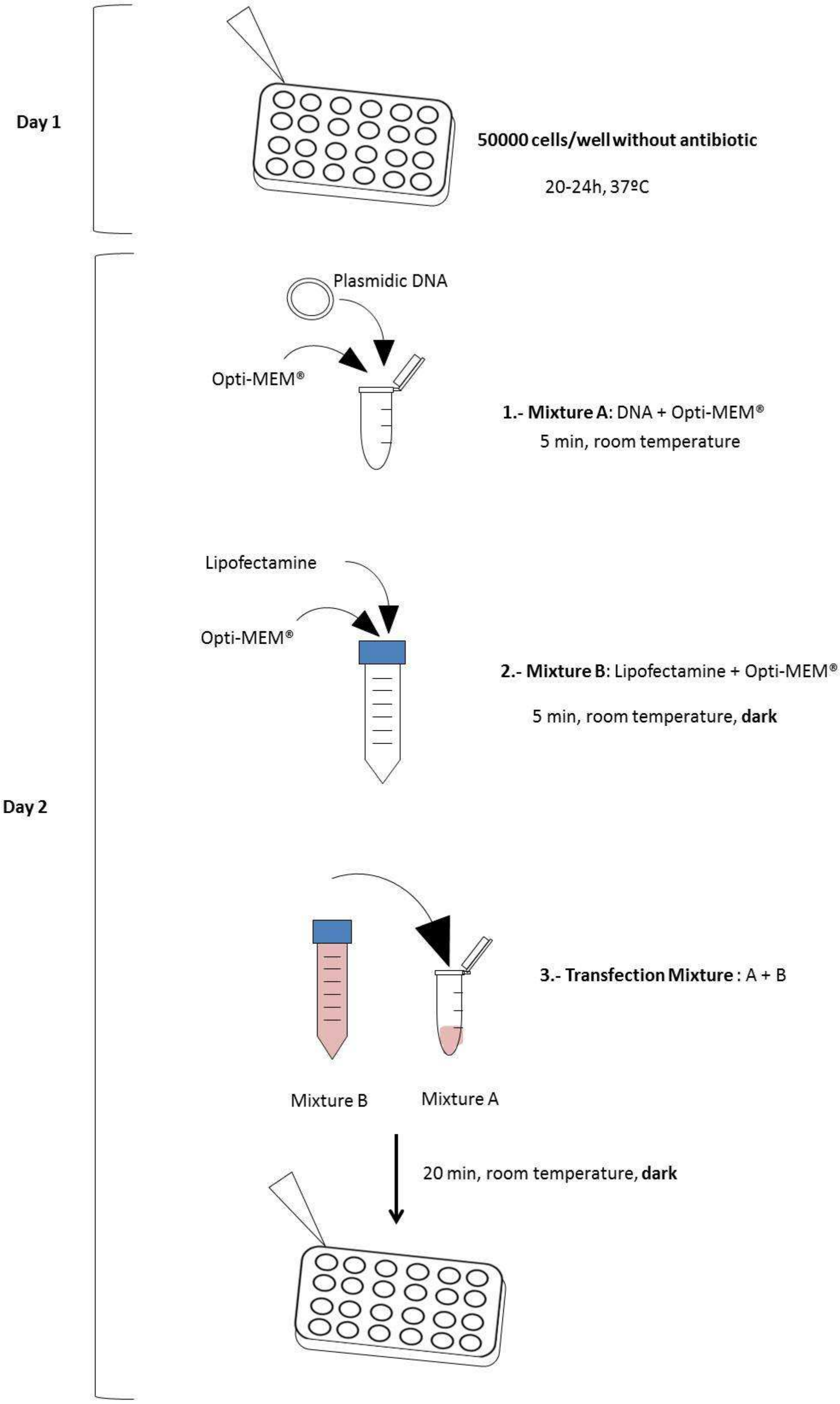

f1 ori
SV40 ori
CMV promoter
pCMV6-Entry Vector
4,9kb
Neo^r/Kan^r
a.
lacZ
pUC18
2,6kb
Amp^r
pMB1 ori
b.
CMVpromoter
PTGDR
pCMV6-PTGDR
Amp^r
c.

Figure 3



Day 1
50000 cells/well without antibiotic
20-24h, 37ºC
Day 2
Plasmidic DNA
Opti-MEM®
1.- Mixture A: DNA + Opti-MEM®
5 min, room temperature
Lipofectamine
Opti-MEM®
2.- Mixture B: Lipofectamine + Opti-MEM®
5 min, room temperature, dark
3.- Transfection Mixture : A + B
Mixture B
Mixture A
20 min, room temperature, dark

Figure legends

Figure 1. Cell density for transfection assays.

Figure 2. Expression vector maps.

Figure 3. Quick protocol for a transfection assay.