CHAPTER 13

TITLE

Cell culture techniques. Corticosteroid treatment in A549 Human lung adenocarcinoma epithelial cell.

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Summary

Experimentation with cell cultures is widespread in different areas of basic science as well as in the development of biotechnology applied to medicine. Cellular models are applied to the study of respiratory diseases. In this chapter we present a protocol of basic cell culture using A549 Human lung adenocarcinoma epithelial cells as model. Corticosteroid therapy is used to treat respiratory diseases such asthma. In this chapter, we present a protocol of lung epithelial cell line culture treated with dexamethasone to illustrate an example of monitoring the effects of a drug in a lung cell culture assay.

Keywords: cell culture, subculture, cell freezing, cell thawing, mycoplasma, corticosteroids.

1. INTRODUCTION

Cell cultures are essential tools not only in basic research, but also in the development of the biotechnology industry, without forgetting its relevance in applied medicine (***1***). They are used in a wide variety of research areas such as virology, cancer research, or immunology, and allow further studies of intracellular activity, intracellular flow, or cellular interactions (***2***). In the study of respiratory diseases such as asthma it is very common to find reports based in cell cultures of human bronchial epithelial cells (***3***), human T cells (***4***) or murine lung fibroblast (***5***), used to analyze the underlying mechanisms as well as the possible development of new therapeutic targets.

There are two different categories of cell culture, the primary culture and cell line. The first are obtained directly from animal tissues having100% of the original karyotype. Cell lines,

however, are formed by aneuploid cells mostly from tumoral origin. Primary cultures have limited lifespan. They grow a limited number of passages before senescing, whereas cell lines have unlimited proliferation and are maintained with several number of passages.

This cellular model of “in vitro” study confers some advantages over animal experimentation: (a) cell cultures provide a controlled environment, allowing a strict control of their physicochemical properties, (b) a large number of homogeneous cells is easily obtained, (c) when possible, the replacement of animal testing by cell cultures may avoid ethical conflicts related to animal experimentation, and (d) cell cultures are more economical than animal models.

However, it has some disadvantages such as: (e) instability, due to their chromosomes aneuploidy cell lines can generate sub-populations when are submitted to a large number of passages, and (f) the cell culture cannot always replace "in vivo" testing, especially in biomedical research.

Corticosteroid therapy is used to treat a variety of inflammatory and immune disorders among others respiratory diseases such asthma. In this chapter, treatment with dexamethasone is used to illustrate an example of monitoring the effects of a drug in a lung cell culture assay.

2. MATERIALS

2.1 Instrumentation and consumables

1. Laminar flow cabinet
2. CO_2 incubator
3. Thermostatic bath

4. Hemocytometer
5. Cell culture vessels (Roux culture flasks, Petri dishes, multi-well plates)
6. 1.5 ml microcentrifugue tubes
7. 15 ml sterile tubes
8. 50 ml sterile tubes
9. Cryovials
10. Automatic micropipettes and appropriate filter tips.
11. Automatic pipettor
12. Dispossable serological pippetes (5 ml, 10 ml, 25 ml)
13. Inverted phase contrast microscopy
14. Centrifuge
15. Microcentrifuge
16. Water purification equipment, balances, pH-meter
17. Ice bucket
18. Sterilization, autoclaves
19. Spectrophotometer
20. PCR Thermocycler
21. qPCR Thermocycler

2.2 Cold storage

1. Liquid nitrogen
2. Electric cold (4ºC, -20ºC, -80ºC)
3. Cryo preservation module (i.e. StrataCooler Cryo preservation module, Stratagene, La Jolla, CA, USA)

2.3 Solutions and reagents

1. Trypan Blue Solution 0.4 %, 0.2 μm filtered
2. Trypsin-EDTA solution (1X) 0.5 g porcine trypsin and 0.2 g EDTA, 4Na per liter of Hanks′ Balanced Salt Solution with phenol red
3. Dimethyl Sulfoxide (DMSO)
4. Dexamethasone: perform a stock solution at a concentration of 2.5 $x10^{-3}$ M in absolute ethanol. Make aliquots and store at -80 ºC.
5. Ethanol absolute ≥ 99.8 %
6. Ethanol 70% (70.14 ml of ethanol 99.8 % and add water to 100 ml)
7. Sterile Fetal Bovine Serum (FBS)
8. Sterile Modified Fetal Bovine Serum like Charcoal Stripped Serum (Gibco, Thermo Fisher Scientific, Waltham, MA, USA)
9. RPMI 1640 medium with L-Glutamine with Phenol Red and without HEPES
10. Antibiotics: Penicilin (100 U/mL)/ Estreptomicin (100 μg/mL). Anti-microbial combination.
11. 1xPBS (Phosphate-Buffered Saline**)**: 1.37 mM NaCl, 2.6 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4. Adjust the pH to 7.4 with HCl.
12. "RNeasy Plus Mini Kit" (Qiagen, Hilden, Germany)
13. Turbo DNAase-free kit (Ambion, Thermo Fisher Scientific, Waltham, MA, USA
14. Master Mix Promega (Promega, Madison, WI, USA)
15. Oligonucleotides: Mico1 5'-GGC GAA TGG GTG AGT AAC ACG-3' and Mico2 5'-CGG ATA ACG CTT GCG ACT ATG-3'
16. Superscript III First Strand Synthesis System for RT-PCR (Life Technologies, Paisley, UK)
17. LightCycler 480 Sybr Green I Master (Roche, Basel, Switzerland)

18. RedSafe Nucleic Acid Staining Solution (iNtRON, Korea)
19. Water, PCR grade

3. METHODS

The methods described below are designed for the A549 cell line from human lung epithelial cells. When working with other cell lines, protocols may suffer some modification. Furthermore, volumes of various reagents are specific for the specified container size and must be adapted in each specific case.

In cell cultures, it is essential maintains aseptic measures to avoid possible contamination to ensure cell viability and the veracity of the experimental results (***6***):

1. Clean the surface of the laminar flow hood with 70% ethanol before experimentation.
2. Clean all those tempered containers in the water bath with 70% ethanol before using.
3. Avoid touching the surface of the mouths of media bottles, reagents and cell containers, pipetting with inclined flask.
4. Loose the caps of all reagents and flasks, but not removes them completely, to facilitate pipetting. Close immediately after us. Keep them open the minimum time possible.
5. Discard any material or culture if you suspect that it can be contaminated.
6. Conduct periodic cleaning of the cabin, incubator and thermostatic bath to avoid contamination.
7. If the cells are grown in culture flasks Roux leave plugs slightly loose to ensure an adequate gas exchange.

3.1 Subculture (passage) of a (non-primary) adherent cell line

For primary cultures, the sub cultivation ratio is 1:2. It is essential to estimate the time required to double the cell population. In continuous cell lines, the sub cultivation ratios can be greater and ascertain the time of "population dubbing" is not so necessary. The standard is passage the cells every 2-3 days at a 1: 3 to 1: 5 ratio. (*See* **Notes 1 and 2**)

The criteria to determine when it is necessary to perform a subculture are as follows (*7*):

1. Cell culture density: it is necessary to make a passage before the cells reach the state of confluence.
2. Impoverishment of the culture medium.
3. Time since last subculture.
4. Especial requirements of the experiment, the need to increase the number of cells or to change the type of culture medium.

3.1.1 Microscopic examination of the cell culture

Although it seems obvious, it is essential to perform a microscopic inspection of the culture before any experimental procedure. Use an inverted phase contrast microscope (100 to 200 X) to quick test the general cell appearance checking that there is no evidence of contamination or dead cells floating in the liquid.

3.1.2 Trypsinization

1. Remove all culture medium of the culture flask Roux 75cm^2 (T-75 flask) where cells are growing.
2. Wash once with 5 ml of Phosphate-Buffered Saline (PBS) to remove traces of Fetal Bovine Serum (FBS) that can inhibit trypsin (*see* **Note 3**). Then slowly rock it back and forth to remove all traces of fetal FBS.
3. Remove the PBS carefully.

4. Add 2 ml of trypsin/EDTA to ensure that the entire surface of the vessel is covered. (*See* **Note 4**)
5. Rotate the culture flask to ensure even distribution of trypsin.
6. Incubate 5 min at 37ºC in the CO_2 incubator to allow cell detaching.
7. Gently tap the tube on the palm of your hand to disperse the cells and check at the inverted microscope to confirm that the cells are detached from the surface. (*See* **Notes 5, 6 and 7**)
8. Add 4 ml (twice that used for trypsin) of pre-warmed complete medium (it must contain serum to neutralize the trypsin). (*See* **Note 8**)
9. Collect the trypsinized cell solution and transfer to a sterile 15 ml tube.

3.1.3 Subculture

1. Centrifuge the trypsinized cell solution, 5 min at ≥ 235 g and remove the supernatant.
2. Resuspend cell pellet in 6 ml of complete medium.
3. Transfer 2 ml of the resuspended cells to a new vial and add 10 ml of complete medium. (*See* **Note 9**).
4. Label the flask with the cell line, date and passage number.
5. Incubate at 37 ° C and 5% CO_2.

3.2 Determination of the number and cell viability.

1. Clean the surface of the chamber cell count or hemocytometer with a solution of 70% ethanol. (*See* **Note 10**)
2. Slightly moisten the side of the camera with water or alcohol and pressing gently slide the cover to affix it.
3. Detach the A549 cells grown in monolayer by trypsinization (see trypsinization).
4. Centrifuge the cells for 5 min at ≥ 240 g.

5. Resuspend cells in 1 ml of complete medium by gently pipetting several times to avoid the presence of cell aggregates.
6. In an eppendorf perform a 1/2 dilution of the cells with Trypan-Blue Solution adding 10 µl of the suspension cells to 10 µl of colorant. (*See* **Notes11 and 12**)
7. Mix well by pipetting up and down to ensure an even distribution, or give a brief vortex and let stand 5 minutes before loading the counting chamber.
8. Take 10 µl with automatic micropipette and load it into the chamber Neubauer cell counting. To calculate the total number of cells takes into account the dilution factor (2). (*See* **Note 13**)
9. Make a first check with 10X and 20X objective to center the counting area of the hemocytometer and verify that the cells are distributed evenly across the surface. (*See* **Note 14**)
10. Focus on one set of 16 corner squares as indicated in blue in the Figure 1.
11. Switch to the 40X objective and count the number of live cells in this area of 16 squares. Count cells that are within the square and the positioned on the right hand or bottom boundary line. (*See* **Note 15**)
12. Determine the concentration of cells per milliliter by the formula:

cells/ml = average count per square × dilution factor × 10^4

Total cells = cells/ml × total volume of cell suspension from which sample was taken

13. Determine the percentage of living cells according to formula (***8***):

$$\%\, viable\ cells\ = \frac{number\ of\ unstained\ cells}{total\ number\ of\ cells} \times 100$$

3.3 Freezing a cell line

1. Check possible contamination before freezing
2. Detach the cells of the culture you want to freeze (see trypsinization).
3. Centrifuge 8 min at ≥ 370 g.
4. Carefully decant the supernatant and resuspend the cells in 2 ml of fetal bovine serum.
5. Perform a cell count using a hemocytometer such as a Neubauer chamber or similar, and performing a vital stain with trypan blue (see determining cell number and viability)
6. Adjust to a final concentration of 5-10 x10^6 cells/ml in fetal calf serum.
7. Put 1.8 ml of the cell solution in a sterile cryovial and add 200 µl of sterile DMSO. (*See* **Note 16**)
8. Freeze immediately at -20 ° C for 24 hours. (*See* **Note 17**)
9. After 24 hours transfer the cryovials at -80 ºC. (*See* **Note 18**)
10. After 24 h, move the cells to liquid nitrogen (-170 °C) for long term storage. Cells are stable at liquid nitrogen for many years.

3.4 Thawing a cell line.

1. Preheat the medium at 37 ºC in a thermostatic bath.
2. Remove the vial from liquid nitrogen tank to dry ice, or from freezer at -80 °C.
3. In a falcon tube, add 30 ml of preheated RPMI in a thermostatic bath.
4. There are two ways to thaw the vial:

    a. Thaw the vial containing the cells in the 37 ºC bath. It is important to thaw quickly to minimize any cell damage. Transfer the cells to the falcon tube with sterile Pasteur pipette. Remember to clean the vial with a tissue soaked in 70% ethanol before opening in the hood, to avoid contamination.

b. With a sterile Pasteur pipette, add tempered medium to the cell vial and transfer them to the falcon tube (This method is preferred).

5. Centrifuge 5 minutes at ≥ 370 g.
6. Meanwhile, prepare a small bottle or plate with 2 ml of complete RPMI, you can put on the stove to be tempered.
7. After centrifuge, discard the supernatant, resuspend the pellet in 3 ml of medium and transfer to the bottle. Pipet gently up and down to avoid cell agglomerates.
8. For sensitive cells, it is recommended to use medium with 20 % fetal bovine serum and to seed in well-plate to be closer.
9. Incubate at 37 °C and 5% CO_2.
10. Examine with inverted phase contrast microscope 24 hours after thawing and subculture if necessary. (*See* **Note 19**)

3.5. Pharmacological treatment with Corticosteroids

Nowadays, one of the most common uses of corticosteroids is the treatment of asthma and other allergic diseases. Thus, inhaled corticosteroids are at the forefront in the treatment of adults and children with persistent asthma (***9***) or COPD (chronic obstructive pulmonary disease) (***10***). In this chapter, treatment with dexamethasone illustrates an example of monitoring the effects of a drug in a lung cell culture assay.

For this test, a modified serum with low concentration in hormones, charcoal stripped serum, is used to supplement the culture medium. This will reduce the potential interference with corticosteroid treatment that could derive from the presence of steroids hormones in a conventional fetal bovine serum.

3.5.1 Cell culture treatment

1. Make a stock dilution of dexamethasone to obtain an intermediate concentration of $2.5x10^{-5}$ M (dilution 1/100) in complete RPMI medium supplemented with 10 % charcoal stripped serum and appropriate antibiotics (*See* **Note 20**).
2. Prepare a 1/100 dilution of absolute ethanol, to treat the cells that serve as basal condition in the experiment. (*See* **Note 21**)
3. Seed A549 cell at a density of 500,000 cells/well in 6-well plates, in a final volume of 1800 µl. Perform three conditions (each in triplicate):
    a. Treatment with dexamethasone to a final concentration of $2.5x10^{-6}$M.
    b. Control of the vehicle (ethanol).
    c. Control treatment (cells alone, without any additives).
4. Add 200 µl of $2.5x10^{-6}$ M dexamethasone to the corresponding treatment wells (the final concentration is $2.5x10^{-5}$M., 200 µl of 1/100 ethanol to the vehicle control wells, and 200 µl of RPMI (10% FBS + antibiotics) to the control wells. (*See* **Table 1**).

3.5.2 Collection of cells

In this protocol we collect the cells at 36 hours.

1. Collect the medium in an eppendorf and store at -80 °C for further determinations.
2. Gently wash the attached cells with 500 µl of PBS.
3. Add 500 µl of trypsin and stored in an incubator 5-6 minutes until cells are detached.
4. Neutralize the trypsin with 1000 µl of complete RPMI medium and transfer the cell suspension to an eppendorf.
5. Centrifuge at ≥ 370 g 5 min.
6. Remove the supernatant and wash the pellet with 500 µl of PBS.
7. Centrifuge at ≥ 370 g 5 min.
8. Remove the supernatant and add 350 µl of the lysis buffer of RNA extraction kit.

9. Vigorously mix the tube on the vortex to disrupt the cells and put away immediately at -80°C until RNA extraction.

3.5.3 RNA extraction

Keep the RNA samples on ice to prevent RNA degradation. Clean the work surface and pipettes used (preferably pipettes designed only to work with RNA) with 70 % ethanol. Make the work as quickly as possible to avoid the RNA degradation.

1. Add the lysate directly to a QIAshredder column and centrifugate 2 min to V max to homogenize.
2. Add 350 µl (1 volumen) of 70 % ethanol to homogenize the lysate and mix well by pipetting. DO NOT SPIN.
3. Pipette 700 µl of the sample to a QIAamp new column. Centrifuge 15s to ≥ 8000 g. If you had more volume, repeat the centrifugation with the rest.
4. Add 500 µl of Buffer RPE to the column and centrifuged at ≥ 8000 g for 15s to wash.
5. Add 500 µl of Buffer RPE and centrifuged at ≥ 8000 g for 3 min.
6. Place the column into a new tube and centrifuged at full speed for 1 min.
7. Transfer the column to a 1.5 ml tube and pipette 30 µl H_2O RNAse free directly on the membrane. Centrifuge at ≥ 8000 g for 1 min to elute the sample.
8. Measure the concentration in Nanodrop. Verify that the 260/280 nm absorbance ratio, which indicates the purity of the extracted RNA, is around 1.8 accepting then the RNA 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 (***11***).

3.5.4 RNA Treatment with DNase

1. Transfer the 30 µl of RNA to a PCR tubes and add:

a. 2.9 µl of DNase Buffer 10X.

b. 1 µl Turbo DNase.

2. Make a brief spin.
3. Incubate 30 min, 37°C.
4. Make a brief spin.
5. Add 3 µl of inactivation resin. Mix well the resin before pipetting it.
6. Incubate the mixture 5 min, room temperature (25ºC). Mix occasionally.
7. Centrifuge 2 min, ≥ 8000 g.
8. Collect the supernatant careful not to catch the resin and transfer to a new 1.5 ml tube.
9. Store the RNA at -20 °C if it is to be used soon, or -80 °C for long periods.

3.5.5 RNA retrotranscription

1. For the retrotransciption, use 1 µg of RNA considering the concentration measured before DNase treatment. Prepare the following RNA-primer mixture:

   1 µg of RNA_____ *n* µl

   50 ng/µl random hexamers___1 µl

   10 mM dNTP mix_____ 1 µl

   DEPC-treated water_____ to 10 µL

2. Incubate the tube at 65ºC for 5 min, then place on ice 1 min.
3. Prepare the following cDNA synthesis mix reaction adding the components:

   10X RT buffer_____2 µl

   25 mM $MgCl_2$_____4 µl

   0.1 M DTT_____ 2 µl

   RNase out (40 U/µl)_____1 µl

SuperScript III RT (200 U/µl)__ 1 µl

4. Add 10 µl of cDNA synthesis mix reaction to each RNA-primer mixture, mix gently and collect by brief centrifugation (*See* **Note 22**).
5. Program the thermocycler as follow:

   25ºC- 10 min

   50ºC- 50 min

   85ºC- 5 min
6. Incubate on ice.
7. Spin for collect the reactions and add 1 µl of RNase H to each tube and incubate at 37ºC- 20 min

The cDNA is obtained in a final volume of 20 µl and an estimated concentration of 50 ng cDNA/µl (***12***).

3.5.6 Perform a quantitative PCR (qPCR).

Work in a laminar flow cabinet.

1. For each sample, 20 ng of cDNA are used, and the following reactions are made:
   a. Three reactions with specific oligonucleotides of gene to study.
   b. Three reactions with oligonucleotides of a housekeeping gene (GAPDH in our experiment).
2. Preparation of samples:

   3.2 µl of cDNA + 36.8 µl of H_2O.Add to each well 5 µl of this sample mixture.
3. Preparation of the reaction mixtures:

   H2O_____1.3 µl

   LightCycler 480 SybrGreen I Master_____7.5 µl

   Primer F/R 10uM_____1.2 µl

Multiply by the number of reactions that will use the same oligos and make a mix for all.

4. Preparation of the plate: Add to each well 5 µl of the cDNA mixture and 10 µl of the corresponding reaction mixture.

3.6 Mycoplasma contamination by PCR

The main source of Mycoplasma contamination in a cell culture is the use of animal serum or trypsin contaminated. Certain mycoplasma species are found in human skin, and poor aseptic techniques can introduced them in the cultures. It is a contamination of small prokaryotic cells that can form colonies, which may go unnoticed since are not as obvious as contamination by bacteria or yeast. Mycoplasma contamination can cause different effects on culture cells altering metabolism and proliferation. Therefore, it is necessary to conduct regular checks of cell culture (***2***).

Commercial kits have been developed to detect the presence of Mycoplasma species responsible for the majority of cell culture contamination by detecting 16S RNA of 8 mycoplasma species (*M. hyorhinis*, *M. arginini*, *M. pneumoniae*, *M. fermentans*, *M. orale*, *M. pirum*, *Acholeplasma laidlawii* and *Spiroplasma mirum*). (*see* **Note 23**).

1. To best results maintain culture cells in absence of antibiotics for several days in order to observe a good signal in the PCR.
2. Take a 100 µl of culture supernatant. This can be store at 4 ºC for a couple of days before used it.
3. Boil or heat to 95 ºC the supernatant for 5min. This step breaks the mycoplasma membrane and releases the DNA.
4. Spin the sample briefly in the microcentrifugue. Transfer the supernatant to a new tube and freeze at -20 ºC for futures PCR analyses.

5. Keep on ice while preparing PCR.
6. PCR conditions:

Primers Mico1/Mico2 (10 pmol/µl each)	1 µl each
Master Mix	12.5 µl
RNAse-free Water PCR-grade	8.5 µl
Sample	2.0 µl
Total PCR volume	25 µl

Program the thermocycler:

95ºC- 12 min

95ºC- 1 min
55ºC- 1 min
72ºC- 1.5 min
} 35 cycles

72ºC- 3 min

7. While the PCR is in progress, perform the 1 % agarose gel in TBE (Tris-Borate-EDTA):
    a. 0.5 g agarose
    b. 50 ml 0.5 % TBE buffer
    c. 1.5 µl RedSafe Nucleic Acid Staining Solution
8. Once the PCR is finished, and the agarose gel is in the electrophoresis chamber, load the PCR sample on the gel and perform the electrophoresis.
9. In the case of a positive sample for mycoplasma, a fragment of 500 bp will appear.

4. Notes

1. Both, the passage time and the cells subcultivation ratio may vary between cell lines or in different conditions. Change the ratios and the time monitoring the cell growth in the inverted microscope preventing a complete cell confluence. 100% confluence may alter the properties of the cell line. In addition, the more confluent the cells the more difficult to disperse into a single cell suspension.
2. In slow growing cell cultures, change the medium every two or three days by replacing 2/3 of the old medium for a new tempered medium.
3. Adjust volumes according to the bottle surface used (**Table 2).**
4. In cultures where cells are well lifted, trypsin can be diluted to the medium with serum free medium or PBS.
5. Adherent cells have a rounded morphology when not attached to any surface.
6. If we check at inverted microscope that some of the cells continue attached on the surface, increase incubation at 37 ºC one or two minutes, be care not to expose the cells for long time so the trypsin action that can damage them.
7. If the cells are 100 % confluent, trypsinization is more dificult because cell-cell interactions prevent the enzyme to reach the interface between the cell and the substrate.
8. Add a double volume of medium with serum then the trypsin volume to counteract the trypsin.
9. Cell lines should be subculture in order to prevent culture dying. Transfer only the appropriate amount of cells for the required cell density (see the ECACC data sheet for the cell line). For A549 split sub-confluent cultures (70-80 %) 1:3 to 1:6 i.e. seeding at 2-4 $x10^4$ cells/cm^2.
10. The surface of the count chamber must be clean and free of dust and watermarks.

11. Living cells look healthy and appear colorless when microscopically observed, while dead cells are blue stained.
12. Pipetting gently up and down to catch cells evenly.
13. Dispense the dilution slowly and check that is positioned between the coverslip and the slide.
14. If the cells are not evenly distributed or cell aggregates are observed, clean the camera and reload after pipetting the initial solution.
15. It is recommended the density being between 20-50 cells per 1x1mm square for counting on camera. If not, perform the appropriate dilution, or concentrate the cells by centrifugation homogenizing in a smaller volume of complete medium.
16. Depending on the requirements of the cell line, the cells can be frozen in DMEM with 20% serum or 100% serum, in any case with the presence of 10% DMSO as cryoprotectant.
17. It is advisable to transfer the cell to a special rack i.e. StrataCooler Cryo preservation module, designed to freeze mammalian cells at a controlled rate of 0.4-0.6ºC/minute to achieve an 80-90% survival rate. In this case transfer the cryovials to the prechilled (4ºC) StrataCooler Cryo preservation module and place it in -80ºC freezer.
18. The cells can be kept frozen at -80°C for 1-2 months.
19. Adjust the volume of the medium and the flask size to achieve the correct seeding density for the cell line.
20. The vial should always be kept at -80°C. At the time of use, remove from freezer, make an intermediate dilution with culture medium and returning it to the freezer to -80ºC as quickly as possible. Always protect from light. The intermediate dilution should not be reused.

21. An assay with the basal condition should be done in parallel to any assay for studying the effect of a treatment. To this, cells should be treated following the same procedure but using the vehicle in which the reagent is dissolved, ethanol in this case.
22. It is advisable to scale up the reaction mixture as needed.
23. There are alternative methods for the detection of mycoplasma, such as ELISA.

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Table 1. Design of dexamethasone assay

	DEXA TREATMENT	**ETHANOL CONTROL**	**TREATMENT CONTROL (CELLS ALONE)**
Cell volumen (500000/well)	1800 µl/ well	1800 µl/ well	1800 µl/ well
Complete medium	-	-	200 µl/well
Dexa 2.5x10^{-5}	200 µl/well	-	-
Ethanol 1/100	-	200 µl/well	-
Total volume	2000 µl/well	2000 µl/well	2000 µl/well

Table 2: Volume of medium according to culture devices

	Description	Surface (cm^2)	Volume (ml)
Flasks	T-25	25	5 to 10
	T-75	75	15 to 25
	T-150	150	30 to 50
Cell culture dishes	35	8	1 to 2
	60	21	4 to 5
	100	55	10 to 12
Multiwell plates	24-well	1.88	0.5 to 1.2
	12-well	3.83	1.0 to 2.4
	6-well	9.40	2.0 to 3.0

Figure 1

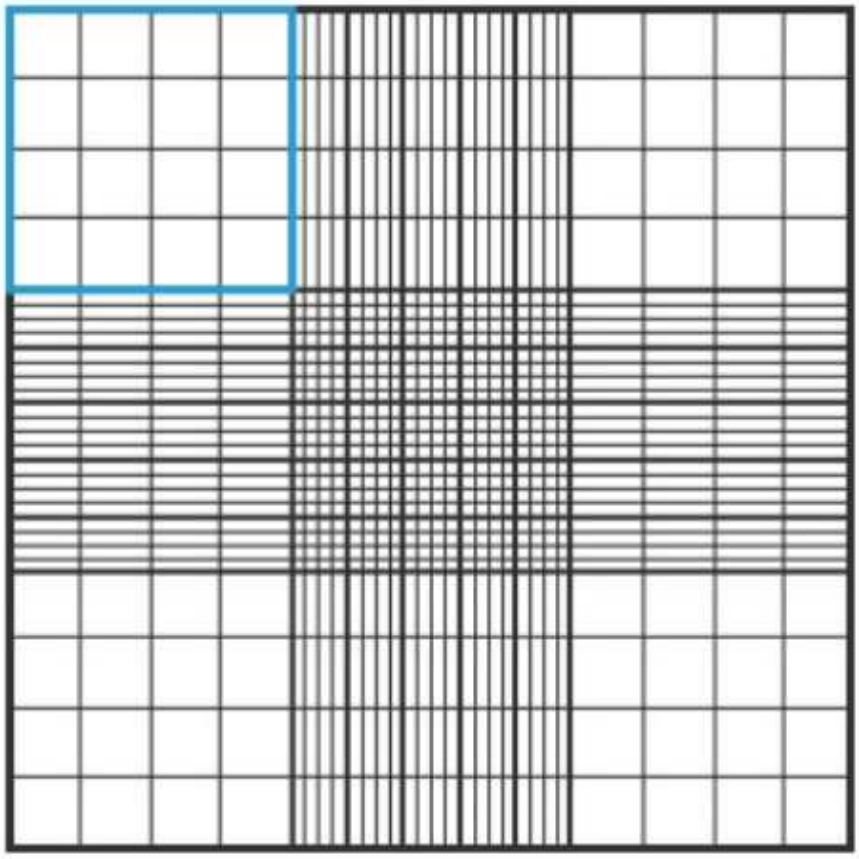

Figure legends:

Figure 1. Diagram of hemocytometer indicating the 16 corner squares used for counting.