Appendix B8

Protocol for the MVLN Assay

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Introduction

The purpose of this method is to characterize the estrogen activity of test chemicals. The assay utilizes an MCF-7[2] derivative that has been stably transfected with the Vit-Luc reporter gene[1]. Thus, the MVLN cell line expresses the endogenous estrogen receptors of MCF-7 and at the same time, contains an exogenous estrogen responsive reporter gene (luciferase). Therefore, the estrogen specific transcription activity of a test chemical is directly related to the luciferase measured in the lysate of treated MVLN cells. The MVLN assay procedure presented here is a modified version of published methods[1, 3]. While this protocol uses the MVLN subclone of MCF-7, all tissue culture materials, such as media and sera, are commercially available. In brief, MVLN cells are seeded into 96 well plates, fed media containing treatment compounds and then two days later, cell lysates are harvested and evaluated for luciferase activity. This general method should also work with other cell lines stably transfected with estrogen responsive reporter systems. When cell counting is called for in this procedure, consult the method "Monolayer Cell Counting with a Coulter Counter" from this laboratory.

Maintenance of Cell Stocks

The MVLN cell line must be obtained from their source[1]. The MVLN clone has been shown to maintain a stable, estrogen responsive phenotype in this laboratory over many passages (at least 50). Stock cultures should be maintained in 10% fetal bovine sera (FBS) media under 5% CO_2 in a 37° incubator. Such culture conditions will be "estrogen rich" and tend to favor cells that require estrogen for growth (MCF-7, MVLN). A regular schedule of passing stocks weekly is recommended. Monday pass into 6 T-25 flasks at a density of 1.5×10^6 for cells to be withdrawn and used for experiments. At the same time, seed 2 flasks at 8.0×10^5 for stock cells. This will be provide enough cells 7 days later to seed 2-3 96 well plates and another round of stocks. MVLN cells may grow slower than other MCF-7 derivatives. In addition, MVLN cells are very sensitive to seeding density. If seeded too light, MVLN cells will grow exceedingly slow and may not thrive. The common pH indicator phenol red has been shown to be estrogenic and therefore should not be used in MVLN cell cultures.

For routine passage, the MVLN cell monolayer is removed with trypsin/EDTA treatment. First, count one duplicate flask. Second, remove media from other flask(s). Then, wash each flask 3X with Ca^{++} free HBSS, remove and then add 2 ml trypsin for 5 minutes @ 37°. After incubation, dilute trypsin to 10 ml with whole media. To disperse the cells, use a sterile cannula-syringe (14 gauge, blunt tip, Luer lock needle with 1 cm at the tip bent 30 to 45°), draw the 10 ml of media up into the 10 ml syringe. Expel the media, with moderate force, through the bent cannula, towards the cell monolayer with a circular motion covering the cell growing surface of the flask. Repeat for a total of 3-5 cycles making sure that all the monolayer has been removed from the flask (keep air bubbles to a minimum). After last cycle, leave the cell suspension in the flask. Then, with a 10 ml pipette, rinse down the inside of the flask 5X with the cell solution.

An aliquot of this concentrated media-cell solution should then be diluted with media in a sterile vessel, mixed and the final volume used to seed flasks/plates. For precise seeding, it is recommended that the entire volume of cells and media to seed all the flasks/plates is mixed in a single vessel. For example, to seed 4 flasks with 1.5×10^6 cells each, make 25 ml of a seeding solution (5ml extra) that is 3.0×10^5 cells/ml, mix well and then add 5 ml to each T-25 flask. The goal of this method is to seed all flasks/plates identical.

Since all MVLN cells can express the reporter gene in response to estrogen, precision within MVLN assays is largely dependent on uniform seeding of plates/wells. It may be a good idea to practice seeding flasks and then count them the next day to check seeding performance.

MVLN Assay Setup and Time Sequence

With the following exceptions, passing MVLN cells for estrogen assays should be done as described above. It is essential that cells used to seed experimental plates have been withdrawn from estrogen 5 days prior to passage. In the example above, a stock flask of MVLN cells grown in FBS media are used to seed 6 T-25 flasks at a density of 1.5×10^6 cells/flask (to be withdrawn) and 2 flasks at 8.0×10^5 cells/flask (stocks) in 10% FBS media on Monday, one week prior to seeding experimental plates. When seeding MVLN stocks or experiments, one of the duplicate flasks is counted to determine the cells per flask count for that series (1 of 2 stocks is counted, 1 of 6 withdrawn flasks is counted). The day after seeding, "stock" flasks (8×10^5 cells/flask) are feed with the same 10% FBS media and fed 5 ml 10% FBS media every other day until used to seed more stock and withdrawn flasks the following week. The remaining 6 flasks are to be withdrawn from estrogens for one week and then passed into plates for experiments. The "withdrawn" flasks are fed the day after seeding with 5 ml 10% DCC FBS media which is almost devoid of estrogens. Two days after seeding, the 6 "withdrawn" flasks are withdrawn from estrogen by rinsing 3X with sterile PBS (all flask surfaces) and fed 5ml of 10% DCC FBS media. This PBS wash process is also repeated on days 3 to 4 after seeding and then these cells are kept on DCC media until used for seeding an MVLN experiment. When using the cannula to disperse cells after trypsin treatment, different cannulas and syringes must be used for withdrawn and stock cells or estrogens will contaminate the withdrawn cells. Keep in mind that it takes a week to get cells ready for a MVLN experiment.

Plates for experiments (96 well) are seeded on day 0 (Monday) with 8×10^4 cells/well in 100ul using 10% DCC FBS media. To ensure uniform seeding, mix the required cells and media in a sterile bottle. Seed the wells using the electronic pipetter set for dispensing 8, 100 µl aliquots. Dispense 100 µl in each well of one plate column with the pipet tip touching the side of the well. Repeat for all 12 columns in each 96 well plate. Mix the cell dilution bottle well before each fill of the pipet for seeding!

Day 1, (Tuesday), cells are fed treatment media. We recommend 4 wells per treatment dose. Treatment media is 10% DCC FBS into which treatments in ethanol carrier have been added. Treatment solutions (2 ml) may be made up in 5 ml polypropylene tubes (do not use polycarbonate or polystyrene tubes![4]) and should be no more than 0.1% v/v ethanol carrier solvent. Higher levels of ethanol may have confounding effects on MVLN studies. Treatment carrier solvents such as DMSO and methanol should be avoided since they may be toxic to cells and/or could have confounding effects on MVLN studies. If DMSO stock solutions must be used, be sure to have proper positive and negative controls in DMSO as well (see below). Media is removed from plate wells using a sterile 8 channel aspirator set with rubber bumpers set such that media only is removed from wells (no cells). Take care to remove media from only 1-3 columns at a time to prevent cells from drying out while adding treatments. Dose wells (100ul/well) using the electronic pipetter set at 4, 100 µl aliquots.

Experimental cells are dosed again the next day (day 2, Wednesday) using the same treatment solutions.

On day 3 (Thursday), treated cells are lysed for luciferase assay. First remove treatment media from each well using a nonsterile, 8 channel aspirator. Then, wash each well 2X with 50 ul PBS. It is essential that all PBS is removed from each well with the aspirator after this step. Thus, removal of the PBS by aspiration is followed by a 1 minute bench top incubation with the plate tipped 45°and a final aspiration of drained residue. To lyse cells, add 25 µl lysis buffer to

each well using the electronic multichannel pipetter and then incubate at room temperature for at least 30 minutes that includes at least 20 minutes on the rotating plate shaker set at 8.

MVLN Assay Design

Properly designed MVLN assays can be utilized to answer only the following 4 questions:

1. Does the test compound stimulate estrogen receptor mediated transcription (what is the shape of the corresponding dose response curve)?
2. If the test compound stimulates transcription, is this response through estrogen receptor mediated mechanisms (is the compound an estrogen receptor agonist)?
3. Can the test compound block the agonist effects of E_2 (is the compound an antiestrogen)?
4. Is the test compound toxic to MVLN cells?

Attempts to obtain additional information from the MVLN assay may be misleading. Keep in mind that this assay does not necessarily determine if the test compound binds to the ER.

Each data point of the MVLN assay should be run in quadruplicate during a trial (4 wells). Then, that same trial should be repeated at least two more times (total of 3 trials).

Example Experiment Setup

Blank (ethanol, same vol. as test cmpd.))	4 wells
E_2 (positive controls) 10^{-12}, 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7} M	24 wells
ICI-182,780 10^{-7} M (check of estrogen free conditions)	4 wells
E2 10^{-9} M + ICI-182,780 10^{-7} M (Check of ICI)	4 wells
Test compound A: 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} M	16 wells
Test compound B: etc.	
ICI 10^{-7} M + Test Comp. A 10^{-5} M (ER mechanism test)	4 wells
ICI 10^{-7} M + Test Comp. B 10^{-5} M etc.	
E2 10^{-9} M + Test Comp. A 10^{-5} M (antiestrogen test)	4 wells
E2 10^{-9} M + Test Comp. B 10^{-5} M etc.	
	to a Total of 96 wells

Note1: Test compound toxicity is determined by comparing Luc activity of test compound treatments and/or test compound with ICI to blank and ICI alone.

Note2: When running more than one plate, the above controls should be used on at least one plate while other plates need only Blank, E_2 (positive controls) 10^{-10}, 10^{-9}M, ICI-182,780 10^{-7} M and E2 10^{-9} M + ICI-182,780 10^{-7} M.

Example Schedule

Day 0 (Monday)	Pass stocks, experimentals plated (from last weeks withdrawn cells)
Day 1 (Tuesday)	Feed all stock flasks (stock and withdrawn for next week) Dose experimental plates
Day 2 (Wednesday)	Withdraw stocks (for next week) Dose experimental plates

Day 3 (Thursday)	Harvest plates and Run Luc Assay
	Feed/withdraw stocks (for next week)
Day 4 (Friday)	Feed/withdraw stocks (for next week)

Regarding an Estrogen Free Laboratory Environment

All glassware, caps, hoses, etc. that may contact media must be free of estrogenic compounds. Soap wash (1% Liqui-Nox), 3X hot water rinse, 3X rinse with ddH_2O, air dry, rinse with 95% ethanol, air dry and then autoclave bottles with caps loosened. Glassware may also be baked at 250° C for 12-24 hrs after ethanol wash. Your cell culture environment should be characterized for estrogen contamination with the MVLN assay treated with and without added ICI-182,780[5]. If the "estrogen free" cells treated with only media have more Luc activity (> 10%) than the ICI treated cells, you have estrogen contamination. All experiments conducted in the presence of such contamination are suspect since regardless of how they are set up, you are testing combinations of estrogenic chemicals. We have found plastic vessels and implements to be the major source of estrogen contamination. Polystyrene and polycarbonate seem to be the big problems[4]. Do not use culture flasks with "phenolic" caps. Filter units may also add estrogenic substances to media. The Corning bottle top units (orange) are suspect. Zap Caps seem to add some kind of nonestrogenic mitogen which induces MCF-7 cells to grow at maximum rate, even in the presence of ICI. It is unclear what effect Zap Cap contamination has on MVLN assays. Also, it appears to be relatively easy to extract estrogens from gloves and/or the hands of females when rinsing items with ethanol. Lastly, ethanol rinsed vessels and implements must be thoroughly dry before use in making media or other procedures.

Media

1. DMEM powder for 10L, (phenol red free, Mediatech 90-013-PB).

k. 59.58 gm HEPES (Gibco 11344-033), media will be 20 mM.

l. 37 gm $NaHCO_3$

3. 100 ml non-essential amino acids (Gibco 11140-019), media will be 0.1 mM.

m. 100 ml sodium pyruvate (Gibco 11360-070), media will be 1 mM.

n. 200 ml L-Glutamine (Gibco 25030-081)

6. 1.0 ml/L media Gentamicin (Gibco 15750-011).

In 2 L tissue culture grade water, add 1 & 2 above. Mix 30 minutes in 3 L beaker. Add 3 & mix 10 minutes, pH to 7.2. Transfer media with 2 L graduate to large mixing bottle and dilute media to total volume of 10 L by quantitatively transferring and washing residue from beaker. Mix 15 min. Check pH and adjust as required. Filter 450 ml into each 500 ml sterile bottle (Gelman VacuCap 4622 or Gelman Micro Culture Capsule 12158). Store media at 4° C.

One 500 ml bottle of media ready to be use on MVLN cells contains: 450 ml DMEM (from above), 5 ml each of non-essential amino acids and sodium pyruvate solutions, 10 ml L-Glutamine solution, 0.5 ml Gentamicin and 50 ml FBS or DCC FBS.

Sera

FBS	Hyclone Characterized Fetal Bovine Sera (A-1115-L)
DCC FBS	Hyclone Charcoal/Dextran Fetal Bovine Sera (A-1120-L)

Buffers

Ca^{++} Free HBSS	Gibco 14185-052
PBS	Gibco 14080-055
Lysis Buffer	Promega E153A

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