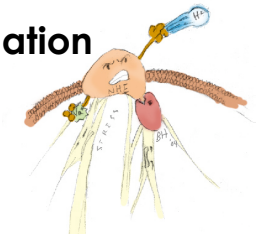
Wallert and Provost Lab

Excel with hard work and inquiry

Soft Agar Assay for Colony Formation

Protocol



Introduction *In vitro* cellular transformation detection assays are semi-quantitative and measure the morphological transformation of cell colonies induced by chemicals. This transformation is associated with certain phenotypic changes such as loss of contact inhibition (cells can grow over one another) and anchorage independence (cells form colonies in soft agar). Anchorage independence can be described in the light of primary fibroblasts and many fibroblastic cell lines (e.g. BALB/c3T3, NIH-3T3, etc.) that must attach to a solid surface before they can divide. They fail to grow when suspended in a viscous fluid or gel (e.g. agar or agarose), however when these cell lines are transformed, they are able to grow in a viscous fluid or gel and become anchorage-independent. The process by which these phenotypic changes occur, is assumed to be closely related to the process of in vivo carcinogenesis. In general there is reasonably good correlation between *in vitro* transformation and *in vivo* carcinogenesis, although the correlation varies depending on the system being studied. These systems are believed to be reasonably good predictors of in vivo activity, and positive results are viewed as potential indications of in vivo carcinogenesis.

The Soft Agar Assay for Colony Formation is an anchorage independent growth assay in soft agar, which is considered the most stringent assay for detecting malignant transformation of cells. For this assay, cells (pretreated with carcinogens or carcinogen inhibitors) are cultured with appropriate controls in soft agar medium for 21-28 days. Following this incubation period, formed colonies can either be analyzed morphologically using cell stain and quantifying the number of colonies formed per well.

Solutions

0.7% (w/v) Agarose (DNA grade)
1% (w/v) Agar (DNA grade)
0.005% Crystal Violet
2X Media + 20% (v/v) Fetal Calf Serum (FCS)

BioReagents and Chemicals

Fetal Calf Serum (FCS)
Agar
Crystal Violet
Agarose
RPMI (or other suitable media)

Suggested amounts for soft agar colony formation assay.

Culture Dish	96 well	48 well	24 well	6 well	35 mm	60 mm	100 mm
Base and Top Agar Volume (mL/well)	0.1	0.2	0.5	1.0	1.5	3.0	5.0
Cells/Well	500	1,000	1,250	2,500	5,000	7,500	12,500
Media volume (ml/Well) for feeding	0.05	0.1	0.25	0.5	0.75	1.5	2.5

Short Protocol

1. Prepare 0.8% base agar layer
2. Prepare 0.7% top agarose solution
3. Harvest cells
4. Count cells and harvest the appropriate number of cells- resuspended harvested cells in top agar
5. Aliquot appropriately on top of base agar layer (pre-warmed to 37°C).
6. Incubate for 10-30 days and feed cells 2 times a week.

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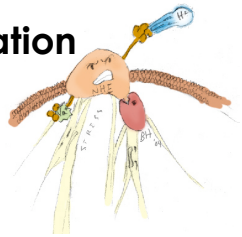

Procedure

A. Preparation of Base Agar – All steps MUST be done sterilely and use cell culture grade water.

1. Melt 1% Agar in a microwave and cool to 40°C in a waterbath (prepare in hood using autoclaved sterile glassware).
2. Using falcon tubes, warm 2X RPMI with 20% FCS* and antibiotics to 40°C in waterbath. Allow at least 30 minutes for temperature to equilibrate. (*Serum may be replaced by agonist, a 1% FCS media or other possible combinations depending on the experimental plans).
3. Mix equal volumes of the two solutions to give 0.5% Agar + 1X RPMI + 10% FCS.
4. Add 1.5 ml of mixture from Step #2 to each 35 mm Petri dish and set aside for 5 min. to allow agar to solidify (**These plates can be stored at 4°C for up to 1 week – let them sit at room temp for 30 min before using**). See notes above for alternative volumes.

B. Preparation of Top Agarose *Note: Certain cell types are sensitive to the percentage of top agar and customer needs to determine if 0.3% or 0.4% agar is best suited to cell type in use.*

1. Melt 0.7% Agarose in a microwave and cool to 40°C in a waterbath (**It is important not to exceed 40°C, otherwise the cells will be killed**). Also warm 2X RPMI + 20% FCS (*see note for serum in media as described above) to the same temperature.
2. Trypsinize adherent cells to release them and count the number of cells per ml. It is very helpful to have a positive control for colony formation. Take care that a single cell suspension is obtained (see below for example).
3. This procedure requires 5,000 cells/plate. By using 20,000/tube, there is enough to plate four agar plates from each original tissue culture plate. Adjust the volume so that the cell count = 200,000 cells/ml.
4. Add 0.1 ml of cell suspension to 10 ml tubes.
5. Label the 35 mm base agar dishes appropriately (from Step 3). (**If they have been stored, it is a good idea to remove the plates from 4°C about 30 minutes prior to plating to allow them to warm up to room temperature**)
6. To plate, add 3 ml of 2X RPMI + 10% or 20% FCS and 3 ml 0.7% Agarose to a tube of cells from Step 4. Mix gently by swirling and add 1.5 ml to each of the three or four replicate plates. Only do one tube at a time so that the agarose does not set prematurely.
7. Incubate plates at 37°C in humidified incubator for 10 to 30 days.
   - Feed cells 1-2 times per week with cell culture media (see above table for volumes).
8. Stain plates with 0.5 ml of 0.005% Crystal Violet for more than 1 hour.
9. Count colonies using a dissecting microscope.

Note: Always include a well containing only base and top agar layers, without cells. This will serve as a background control for cell quantification.

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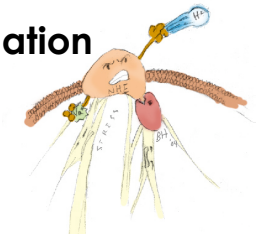

Positive Carcinogen Control Initiator:

1. Treat a fibroblastic cell line (e.g. BALB/c3T3) with 500ng/mL of NMethyl-N'-nitro-N-nitrosoguanidine (MNNG) for 72 hours. Note: Exercise extreme caution when working with carcinogens! Wear gloves, protective clothing and facemask.
2. Remove MNNG-containing media and culture cells for 3 days in normal conditioning media without additives.
3. Culture cells for 2 more weeks in conditioning media containing 100ng/mL of 12-O-tetradecanoylphorbol 13-acetate (TPA).
4. Following TPA treatment, culture cells one more week in normal conditioning media, without any additives.
5. Continue with soft agar assay.

***In Vitro* Cell Transformation Results**

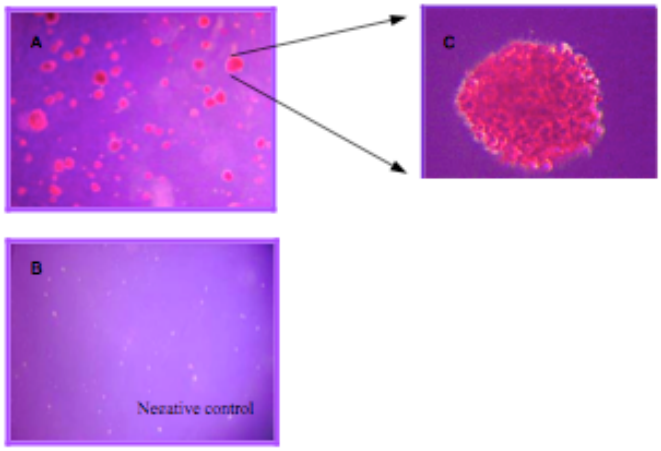

Figure 1. Colony formation revealed by cell stain solution. Low magnification showed **(A)** large colony formation for A549 (lung carcinoma) cell line and **(B)** no colony formation or live cells visible for MRC-5 (normal lung fibroblast) cell line. **(C) High** magnification revealed an A549 colony stained with our cell stain solution.