Soft agar assay protocol

Before proceeding, please watch the video in the link provided below as it provides a useful visual run-through of the entire method: https://pubmed.ncbi.nlm.nih.gov/25408172/.

Base agar

Prepare prior to starting protocol:

1. 10mL complete media in Falcon tube
2. 6-well plates

Method:

1. For base agar, prepare a 1.2% agarose solution by adding 1.2 g agarose to 100mL milliQ water to a sterile 200mL Schott bottle.
2. Microwave for 2.5 minutes or until all of the agar is dissolve. Caution: The bottle will get very hot at this point, be careful when handling.
3. Bring the solution into the tissue culture hood, spray the outer layer of the bottle generously with 70% ethanol.
4. Let solution cool down for one minute, gently swirling in the hood. While doing so, it is important to avoid bubbles forming in the solution.
5. Add 10mL of the dissolved agar into the Falcon tube with 10mL media and invert gently. Again, it is essential to avoid or minimize bubbles in the solution. This will form a 0.6% base agar mix.
6. In each well of a 6 well plate, slowly and gently aliquot 2 mL of the agar+media solution to form the base agar. Note: You want to pipette 1mL extra for the number of wells you want to fill. For example, if you want to fill 4 wells, pipette (4 wells * 2 mL) + 1mL = 9mL solution. If you do 8mL, you will form a massive bubble in the last well.
7. Let the solution set in the hood for 15 mins. You can wrap this in a bag and place at 4 degrees Celsius overnight.

Top agar

Prior to starting protocol:

1. Place the base agar plates prepared earlier in the hood and bring it to room temperature.
2. Bring in heatblock into the TC room, set the 15mL block at 37 degrees Celsius.
3. Prepare and label empty Falcon tubes according to experimental design (e.g., control, knockdown/out, overexpression etc.).
4. Trypsinize cells long enough to generate a single cell solution (this might take a few more minutes than the usual trypsinization procedure), resuspend in media, and count (preferably manually). Prepare a 3mL solution of 1,000 cells/mL stock.
5. Aliquot this stock solution into the respective labelled 15mL Falcons, and place tubes in the heat block.

Method:

1. Prepare a 0.6% agarose solution by adding 0.6g agarose to 100mL milliQ in a 200 mL Schott bottle. Microwave until agar dissolves.
2. Bring the solution into the tissue culture hood, spray the outer layer of the bottle generously with 70% ethanol.
3. Aliquot 5mL of the agar solution into 15 mL Falcons, and immediately place in the heat block.
4. Wait for 2-3 minutes, the agar can cool quite quickly, so this part might require some judgement.
5. Bring the cooled agar and cell stock of desired experimental condition into the culture hood, and add 3mL agar (from the 5mL aliquot) into the 3mL of cell stock. Slowly and gently invert, avoid bubbles. This will form a 500 cells per mL solution.
6. Gently aspirate (adjust the pipette setting if need be) 5mL of the stock-agar solution and add 2mL into two wells of a six well plate, thereby adding a total of 1,000 cells per well in technical duplicates. Thus, you will have 1mL left not plated, dispose this.
7. Repeat steps 5 and 6 for the remainder of the experimental conditions.
8. Let the top agar set for 20 mins.
9. Add complete media before placing the plates in the incubator.
10. Replace media every 3 days.

Note: The cell numbers and top agar percentage may require optimization. A good start would be to see if someone has published this information. Very low cell numbers may result the colonies not growing at all, too high (dense) will result the single colonies growing into each other. Too high percentage agar may ‘crush’ cells, too low may allow cells to migrate through to the sides and bottom of the well.

End point

1. After one month (or lesser, depending on experiment), remove media and gently rinse with PBS.
2. Add a fix and staining solution (0.0025% (w/v) crystal violet + 800mL milliQ + 200mL methanol).
3. Incubate for 30 mins at room temperature.
4. Remove stain and wash several times to remove as much background possible.
5. Manually count colonies per condition.
6. Scan plates using a scanner (remove any solution prior). Dr. Wan Lixin lab at Moffitt has a good scanner for this purpose. Prepare image for presentation/publication.