Lazzara Lab
Alice M. Walsh & Janine Buonato
Modified by MJL June 18, 2017

Soft agar colony formation assay

Adapted from protocol provided by Mark Greene's lab (UPenn)

- Seaplaque low-melting temperature agarose, $112.10/25g (Cambrex Biosciences or Lonza)
- Make ahead complete DMEM (with 10% FBS, pen/strep) + HEPES: Add 2.5 mL 1M HEPES to 100 mL complete DMEM. Always feed cells with complete DMEM+HEPES.

1. Set water bath to 42ºC. Autoclaved agarose will be placed in this bath.
2. Autoclave 3% Seaplaque agarose in HBSS (example = 10 mL HBSS + 0.3 g agarose). Autoclave at 121ºC for 15 min, liquid cycle. Place autoclaved solution in 42ºC water bath.
   - *Note that you can make an agarose stock to use for multiple experiments to help reduce variability in agarose concentration. If agarose is already made, microwave it just until you have an evenly melted solution, and place it in the water bath.*
   - *Note also that some protocols call for the preparation of 5% agarose, for a final top layer agarose concentration of 0.5%.*
3. To pour a bottom layer in plates at 0.6%:
   Heat 40 mL media and agarose stock in 42ºC bath. Mix 40 mL media and 10 mL agarose (invert conical tube carefully) and dispense 1.5 mL per 35 mm plate (or 3 mL per 60 mm plate). Remove any big bubbles by aspirating with a Pasteur pipet. Allow the mixture to harden in incubator for 1 hr or more. Put plates quickly in cold room for 5-10 min to set.
4. Move agarose to 37ºC while prepping cells to allow it to cool from 42ºC; this will enhance cell viability.
5. Prep cells in regular 10% FBS media. For each cell type or condition, make tubes with 2.5 mL and 2.5×10^4 cells (for final density of 7500 cells per 35 mm plate). Put in 37ºC bath to equilibrate temperature.
6. Take out one cell type at a time and add 2.5 mL 0.6% agarose/media solution. This will make the final top layer a 0.3% agarose solution. Plate 1.5 mL per plate; with 5 mL total of cell/agarose mixture, you have enough for 3 plates.
7. Allow the top layer to harden for 1 hr in hood and then transfer to 4ºC (cold room) for 15 min before putting in incubator in a single layer.
   - *Note that if incubator temperature is not properly calibrated the gels may fall apart due to gel melting. Even a one degree temperature difference can be important over the weeks' long experiment.*
8. Feed 0.3 mL media the next day and then re-feed ~0.3 mL every three days, enough to prevent the gels/cells from drying out but not so much that liquid accumulates.
9. When colonies are visible (~ 3 weeks), stain with crystal violet and image on gel imager with bright light filter. See staining details below.

For GBM cells:
- plate 5000 cells/35 mm plate
- image after 3 weeks (longer may be necessary to see larger colonies for some cells)

Staining colonies with crystal violet

1. Count colonies on each gel *before* staining. Count twice. Divide plates into quarters to help with high counts.
2. Add 0.3 ml of 0.1% crystal violet solution to each plate.
   0.1% Crystal Violet solution = 3 mL 0.5% crystal violet + 1.5 mL ethanol + 10.5 mL water
3. Incubate 15-20 min on a level surface. Rotate gels. Can see dye diffusing through gel. It is OK if it has not reached the bottom by 20 min, dye will continue to permeate gel. After this be sure to work on top of benchtop soaker, dye solution will spray out and stain everything.
4. After staining, carefully add 1 mL water to each plate. Let sit 5 min.
5. Aspirate the wash CAREFULLY. With all the washes gels will become very loose. For aspirating, don't move pipet tip down; just leave near top of the plate against the wall and let liquid slowly aspirate. Do not tilt the plate toward aspirator pipet as usual, leave the dish flat.
6. Do 4-5 washes the same way. You can image the same day. Best results seem to be when you leave dishes overnight in cold room with a final 1 mL wash.
7. Take pictures on a gel imager using a white light filter. ~0.4 s exposure seems to work well.