Dave Knecht's PROTOCOL FOR UNDER AGAR ASSAY

1. Pour 14 ml of agarose into a 100-mm petri dish or 8 ml in a P60 dish

1a. Larger amounts of agarose may be used **(I have use 25 ml. It doesn't seem to matter how much you use up to a point, DAK 11/01).** Smaller amounts become difficult to work with. The actual volume and thickness seems to have no effect on behavior. The petri dish is fine for imaging with an inverted microscope with a long working distance objective. For higher resolution imaging we use either Rose Chambers, or Willco Wells glass cover slip bottom dishes. The volume is adjusted so the same thickness of agar is formed. Wells are cut with the same design with a razor blade as below with the Willco Wells. With a smaller Rose chamber, the wells are cut with a short razor blade (10 mm long).

1.b Different types of agarose may be used. We routinely use FMC Seakem GTG gel agarose. The agarose can be made up in a variety of media. We routinely use a nutrient (SM) but non-nutrient, buffer, and other nutrient agaroses may be used. When I used an HL-5 media, I distinctly saw less movement but it did not stop all migration. If you try to use just buffered agarose, the agar sticks very tightly to the dish and there is no movement out of the wells.

Recipe for SM:
10g Difco Bacto Peptone
10g Glucose
1g Yeast Extract
1.9g KH_2PO_4
0.6g K_2HPO_4
to 1L dH_2O
pH=6.5

1c. Different percentages of agarose may be used. I use 0.5% for my "permissive" environment and 2% for my "selective" environment. Above 3% the cells don't generally move. Percentages below .5% become difficult to work with. The SeaKem GTG is particularly good for its workability at the lower percentages.

1.d I autoclave the amount of agarose I need for each assay as I need it. The solution is sterilized for 5 minutes and then set on slow exhaust for 5 minutes for the sake of reproducibility. I found that when I make up large amounts and save it, and melt it per use, the motility of the cells decreases over time. A noticeable carmalization takes place after multiple "meltings." The percentage of the agarose probably increases as well. The agarose is poured immediately after removal from the autoclave.
(I use a microwave to melt the agarose solution in sterile SM and that has worked fine, DAK 11/01)

1e. Allow the agarose to harden in a humidified chamber. I leave the petri dishes in this chamber throughout the assay. About 10-15 minutes is usually sufficient. (**I usually pour the dishes the night before the assay and leave them at room temp overnight. DAK 11/01)**

2. I print out a template drawn for the cutting of the trough showing the outline of the dish and the three troughs: The troughs are 2mm wide and 39 mm long (the length of a razor blade). Three troughs are placed 5mm apart in the center of the dish.

   I cut my troughs 2 mm wide and 5 mm apart using the template. I use a new, or almost new, standard single edge razor blade to cut the troughs. The reason for the "newness" of the razor blade is due to the consistency of the trough edge. A major imaging concern arises here. You need to hold the blade as perpendicular as possible when cutting the agar. An "edge effect" occurs due to the skew of the cutting angle which makes imaging near the trough edge very difficult due to light contrast. After the edges are cut, I use a pasteur pipette to "cut" the ends of the troughs. Then the pipette is attached to suction and the troughs are aspirated. Care needs to be taken so as not to lift the edges of the troughs. (I do this by taking a microscalpel, cut the ends of the trough and then lift the agar slab with the scalpel until I can grab it with my fingers, DAK 11/01)

3. The chemoattractant is then placed in the center trough. The trough can accommodate about a 100 ul volume of 0.1mM folate. (I use thicker plates and 200ul of folate, DAK 11/01)

   100mM Folic acid stock preparation

   a) Prepare100 mM stock solution in water and add NaOH dropwise until it dissolves. (For 10ml add 0.44g folic acid and add 10N NaOH until most of the solid folic acid dissolved, then add drop by drop of 1N NaOH until everything dissolved. pH is around 7.8)
   b) You can filter it or it may be self-sterile at that concentration.
   c) Store frozen and dark. (for 2-3 weeks)

   Rifkin recipe- Dissolve folic acid in 0.1N NaOH to 50mM and store dark and cold. This is a 500x stock for under agar. Dilute in KK2 or other buffer for use. According to Rifkin it is stable for at least 1 year at 4C in dark.

4. Allow about 1 hour for the gradient to develop and diffuse to the peripheral troughs (I add the folate and then add start prepping the cells and add them whenever they are done (usually around 20-30 minutes. I could not see any reason not to have them there ready and waiting when the gradient arrives, DAK 11/01). The gradient is still present up to 9 hours after the application of the chemoattractant.

5. I found that cells used in log phase of growth are the most mobile. To obtain these, I plate cells at 4 x 10^5/ml in 10 ml of HL-5 in 100mm petri dishes 2 days prior to the beginning of the assay. This way the cells are still in log phase, barely, on the second day. I also found centrifuging the cells down was detrimental to motility. The assay still works, but not as robustly. Therefore we grow the cells to a usable density, harvest and plate directly without centrifugation. (I have found the centrifugation to be a minor issue, and as long as all cells are handled equivalently, it still works fine. I just make sure they are growing, log phase cells, which does matter, DAK 11/01).

6. Adjust the cells to a density of 10^6/ml in HL5, and then add 100ul to the troughs (I use 200ul with the same number of cells. There is less of a meniscus effect by increasing the volume, DAK 11/01). Higher densities may be used but analysis of individual cells becomes difficult.

For aesthetic purposes, in order to visualize a uniform "front" of cells, up to 10^7/ml may be used. At lower densities much fewer cells migrate out of the trough. It appears that if a cell is not very near the trough edge, it does not sense the gradient.

7. The first few cells usually start to exit the trough within an hour and can be imaged over the next 6-8 hours moving in the gradient.