

Obtaining clones from the wild to freeze away

Plating out samples

Plating out these samples is a compromise between nutrients dilute enough to discourage fungi and rogue bacteria, but strong enough to feed the *Klebsiella aerogenes* that are for our social amoebae. We also need to process many plates at a time. The compromise we have reached is a soil dilution of about 15x, and a weakly nutrient plate with lots of bacteria.

The goal is to see, identify, isolate and grow up alone as many clones as possible, getting them into the freezer quickly and efficiently.

Have ready:

25 labeled hay infusion petri plates (labeled on agar side), 5 per soil sample	Ka solution
Bunsen burner	Pipetteman and tips
Alcohol and spreading rod	
Balance	Paper for list
	Soil samples

The *Klebsiella aerogenes* solution should be a stationary liquid culture. Be sure to grow new Ka from a single spot clone frequently, and avoid any mucoid clones. (learn about correct bacterial culturing elsewhere, but be sure to have enough of your own bacteria.)

Hay infusion medium

per liter: 3 g hay from leached dried hay (largely *Poa* spp.) let sit overnight, for an hour, or microwave, then filter through cheesecloth if necessary. Make back up to 1 liter volume (or whatever you started with) with distilled water. Buffer this infusion with 1.5 g KH_2PO_4 and 0.62 $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ per liter to yield pH 6.0. add 15 g agar/L

1. Plating out the dirt:

- for each soil sample from a particular location prepare **5 hay infusion plates**:
 - label the plates on the agar side with the assigned number, the soil sample number, the date, your initials and an acronym of your choosing to represent the location
 - record all location information in your notebook; it is a good idea to set up a chart for tracking the plates in your notebook as well, making sure to note the soil sample number for each plate (see posted sample sheet)
 - once labeled, pipette **400 microliters of KA** onto each plate
- prepare the dirt solutions:
 - for each soil sample measure out **5g of dirt** and mix with **45g (45mL) of water** (if you do not have enough soil to use 5g of it, use less but make sure you still have a **1:9 ratio of soil to water in your broth**)
 - swirl the broth and let it settle
 - take **5mL of the resulting soil broth** and mix with **7.5mL of water**
 - swirl the broth and let it settle

-pipette **500 microliters** of the final broth onto each plate designated for that soil sample

-spread the KA and broth solution evenly on each plate, taking care not to contaminate between soil samples (flame the spreader between soil samples; it does not need to be flamed between plates of the same soil sample)

-leave the lids of the plates slightly ajar until all the liquid has been absorbed and then store your plates in your drawer.

2. Check plates 2-3 days later and every day or two for 6-10 days

-with a colored marker circle and annotate areas of growth on the lid of the plate, record observations in the chart in your notebook. To help you keep track of the growth use a different colored marker for each day. Make a line on the plate edge so you can align the top and the bottom of the plate. Some species are tiny; some come up later than others. Look hard.

-pick up slugs and fruiting bodies from the plates:

-for each growth picked up put the fruiting body or slug down in the middle of a **300 microliter strip of KA** bacteria solution on a **starving plate**.

-label the plates with the date, the location, number of the plate and then a letter A-Z corresponding to the area of growth (for example, the first fruiting body picked up would go on plate A, the next B and so on)

-make sure this is all recorded in your notebook as well.

3. When the starving plates have grown to yield fruiting bodies:

-check for contamination (mainly that fibrous looking fungus)

-if the plate is **still contaminated** then pick up the cleanest looking fruiting bodies and plate them on a **new starving plate with a 300 microliter KA strip**. Keep repeating this process until the fruiting bodies look clean.

-if the **fruiting bodies look clean** then move ahead to grow them clonally (step 4)

4. Growing clonally:

-from the clean starving plate **pick up a single fruiting body** and place in a 1.5mL eppi tube with **1mL of KK2**. **Vortex**.

-from that spore solution take **10 microliters** and place it into another 1.5mL eppi tube with **990 microliters of KK2**. **Vortex**.

-from that final spore solution take **10 microliters** and place on a labeled plate (ID, date, your initials, "clonal" or whatever designation you see fit) with the appropriate amount of KA.

-if you are using **hay** plates for this step use **200 microliters of KA**

-if you are using starving plates use **500 microliters of KA**

-spread evenly.

-check clonal plates in 2-3 days. I usually find 2.5 days or around 60 hours optimal. If you see aggregation or fruiting bodies you are too late!

5. Plating from clonal plates:

-look for single colonies on the plates (circles)

-using a **yellow pipette tip**, take up some of the cells from a single colony.

-place the pipette tip with the cells in an eppi tube with **300 microliters of KA**

- using the pipette aspirate the cell/KA solution. **DO NOT VORTEX CELLS**
- plate out your cell solution onto a starving plate labeled appropriately
- 6. When the full clonal plates have grown** to yield fruiting bodies:
 - if the plates **look contaminated** use the method described in step 3 to clean then up and then when they are clean move on to the step 7
 - if the plates **look clean already then move on to step 7.**
- 7. Plating out to freeze away:**
 - collect several fruiting bodies from the previous plate and place in an eppi tube with **300 microliters of KA. Vortex.**
 - plate out the spore solution on a **SM plate** that is appropriately labeled. **Spread evenly.**
- 8. When the full SM plates have grown:**
 - if contaminated, clean up as previously described (starving plate and 300 microliter KA strip) and then repeat step 7 again.
 - if **clean then freeze away.** The protocol for freezing away samples is online at www.ruf.rice.edu/~evolve.