Quality Control Procedures of the Chlamydomonas Center

Culture Media

Nearly everything is kept on Sueoka's high salt medium with added acetate and yeast extract (we call this YA medium). The reasons for this are a) all the strains will grow on it, including auxotrophs like arg and nic mutants; b) it's easy and relatively cheap to make; c) it's an enriched medium, so if you get contamination with fungi or bacteria, it will be apparent immediately.

This last point is very important. Bacteria can grow along with the Chlamy on acetate or minimal medium without being obvious. Some bacterial contaminants are very hard to get rid of, because they are sticky or rapidly motile, so it is far better to avoid getting them in the first place. Fungi are usually apparent on YA or acetate medium, but can lurk on minimal medium without showing up to a casual inspection.

The few exceptions are as follows:

The dark and dim strains on YA do better and last longer with twice the concentration of acetate. They do better on plates than in tubes.

The *osm* mutants require TAP medium with either sucrose or lactose. They do fine in tubes on this medium.

A very few strains, mostly other species of Chlamy, don't seem to like acetate and do better on minimal medium. They do OK in tubes, but need to be watched carefully; once they start to fade, they expire quickly.

Tubes are made with 10 ml of agar and slanted when they come out of the autoclave. This provides a bigger surface area from which you can remove loopfuls of cells for sending or transfer. Stab cultures are admittedly a little quicker to transfer, but they don't give you nearly as good a volume of available cells to sample from. Also, it's easier to inspect slant cultures to see whether they need to be transferred soon.

To make slants, prepare 1 liter of medium in an Erlenmeyer flask and autoclave just long enough to melt the agar. With our autoclave, it takes 12 minutes (plus slow exhaust, of course). Although you can melt the agar on a hot plate with a stirring bar, it's the proverbial watched pot, and as soon as you turn your back on it for a second, it will boil over. Dispense 10 ml aliquots into the tubes using the motorized pipetter. This goes faster if you use a 10 ml pipette with a broken tip. Cap, autoclave for 20 minutes, then prop up the racks to create the slants. It takes some practice to get exactly the right angle; what you want is for the agar to reach to about an inch below the top of the tube. Let the tubes age for a week or more before using them, otherwise the agar surface will be wet and the Chlamy don't seem to like this.

Minivials for shipping contain 1.5 ml agar, and are also slanted. Use the big metal rack for making these. It holds 90 vials. Make these as part of a liter from which the rest goes into slants.

YA medium

1) Beijerinck's solution [in the kits that we send out, this is called simply "salts solution"]

NH_4Cl	100 g
$MgSO_4 \cdot 7H_2O$	4.0 g
$CaCl_2 \cdot 2H_2O$	2.0 g

water to 1 liter

For the sulfur-free salts solution distributed with the hydrogen project kit, substitute 3.30 g $MgCl_2 \cdot 6H_2O$ for the magnesium sulfate.

For N- medium for gametogenesis, omit the ammonium chloride

2) Phosphate solution

K_2HPO_4 (anhydrous)	288.0 g
KH_2PO_4	144.0 g

water to 1 liter

3) trace elements (see below)

For YA tubes:

5 ml Beijerinck's
5 ml of phosphate
1 ml of trace elements
1.2 grams sodium acetate (anhydrous); for the hydrated salt use 2.0 g
4 grams yeast extract (Difco)
20 grams agar

For plates, use 15 grams agar per liter

Keep the stock solutions in the refrigerator. I've never had a problem with them getting contaminated.

Hutner's Trace Elements

[Hutner et al., Proc. Am. Philos. Soc. 94: 152-170 (1950)]

For 1 liter final mix, dissolve each compound in the volume of water indicated. The EDTA should be dissolved in boiling water, and the FeSO4 should be prepared last to avoid oxidation.

EDTA disodium salt	50 g	250 ml H_2O
$ZnSO_4$. 7 H_2O	22 g	100 ml
H_3BO_3	11.4 g	200 ml
$MnCl_2$. 4 H_2O	5.06 g	50 ml
$CoCl_2$. 6 H_2O	1.61 g	50 ml
$CuSO_4$.5H_2O	1.57 g	50 ml
$(NH_4)6Mo_7O_{24}$. 4 H_2O	1.10 g	50 ml
$FeSO_4$. 7 H_20	4.99 g	50 ml

Mix all solutions except EDTA. Bring to boil, then add EDTA solution. The mixture should turn green. When everything is dissolved, cool to 70 degrees C.

Keeping temperature at 70, add 85 ml hot KOH (20%).

Cool to room temperature and bring to 1 liter final volume.

Usually the solution will be clear green initially but will turn dark red or purple over the next few days and leave a rust-brown precipitate. To accelerate the “maturation” process, bubble the solution with air overnight on the benchtop. If no precipitate forms, or the solution remains green, check the pH. It should be about 6.7. If it's radically off this, try adding either KOH or HCl to adjust it.

The ritual calls for stoppering the flask with a cotton plug (to allow air exchange) and swirling it once a day for 1 to 2 weeks.

Filter through two layers of Whatman #1 filter paper; repeat if necessary until the solution is clear.

The final product will approximate a good Bordeaux in color. However, even a subtle shift in pH can cause a color change. We had one batch that looked like grape Koolaid but worked fine.

Store refrigerated or frozen in convenient aliquots. This can be made in larger batches - 2 or 3 liters - if you prefer.

Sulfur-free trace elements

For 1 liter final mix, dissolve each compound in the volume of water indicated. The EDTA should be dissolved in boiling water.

EDTA disodium salt	50 g	250 ml H_2O
$ZnCl_2$	10.0 g	100 ml
H_3BO_3	11.4 g	200 ml
$MnCl_2$. 4 H_2O	5.06 g	50 ml
$CoCl_2$. 6 H_2O	1.61 g	50 ml
$CuCl_2$. 2 H_2O	1.00 g	50 ml
$(NH_4)6Mo_7O_{24}$. 4 H_2O	1.10 g	50 ml
$FeCl_2$.4 H_2O	3.60 g	50 ml

As for the usual trace elements recipe, mix all solutions except EDTA. Bring to boil, then add EDTA solution. The mixture should turn green. When everything is dissolved, cool to 70 degrees C. Keeping temperature at 70, add 85 ml hot KOH (20%).

Bring the final solution to 1 liter total volume.

The color is the same as the original trace mix.

Preventing Loss of Cultures

These recommendations are based on the combined 80 years of Chlamydomonas culturing experience from Drs. Harris, Lefebvre and Silflow.

Maintain two complete sets of cultures, preferably in locations sufficiently far apart that a fire or other disaster striking one would be unlikely to hit the other. Designate one set as "primary", and use that as the source for the cultures you send out.

Install high-temperature cut-off switches that will turn off the lights if the air conditioning fails.

For each set of cultures, keep tubes in order in racks. Make a chart or numbered list, so that if a space is open in a rack, you know what tube belongs there.

For cultures kept on plates, put a piece of foil on the shelf and stick on tape or adhesive labels to indicate what strain belongs on each spot. Again, if there's a blank space on the shelf, something is missing.

The sooner you realize something is missing, the more likely it is you will find it.

Inspect the cultures on a regular schedule (see next page).

Nobody uses stock center cultures directly except the stock center technician. If other people in the lab need strains, they ask you and you make plates for them. Make sure everybody in the lab knows this rule, and that it is enforced.

A corollary to this is that if someone takes a stock center strain and modifies it, crosses it, or otherwise alters it, it should never be entered back into the collection under the same stock number.[1]

Use pre-printed labels. If you have to transfer a culture and no label is made up, write the stock number on the patch on the tube itself, not just on the cap. But put a label on as soon as you can!

Put a circular label on the top of every tube when you transfer it. Use a color-coding system that indicates the month of transfer. Although some of these labels will fall off in time, and they are admittedly a bit messy, we've found they are worth the effort. First, they make it easy to spot a particular tube in a rack. Second, you will get an idea from the transfer dates just how well your cultures are doing, which ones need to be transferred more frequently, and whether a culture that looks weak is getting old or just hasn't had time to grow up yet.

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[1] case history: a Duke student once took strain CC-373 ac-u-c-2-21 mt+, crossed it, selected the same mutation in mating type minus, then called that strain CC-373 mt-. Eventually someone else got this from him. with an incomplete history, transformed it with a plasmid and tried to cross one of her transformants to a wild-type mt- strain. Since she assumed it was plus as indicated in the stock list, she spent some months of frustration thinking her mating reactions weren't working, or it had acquired a new mutation, until somebody suggested she test the mating type. There are several lessons to be learned here.

Transferring Cultures

Until you're familiar with the collection and know how cultures will behave under your conditions, I strongly recommend that you inspect every tube and plate in the collection once a week. Once you're sure everything is growing well, and that you aren't having any problems with contamination, then you can go to a schedule of every two weeks, alternating between the primary and backup sets of cultures.

Cultures on plates should be inspected more frequently, since these are more vulnerable to fungus contamination than those in tubes, and are also likely to be less healthy.

Cultures need transfer if they are

changing from bright/dark green to olive drab
getting very pale
starting to bleach out at the top of the agar
drying out (agar shrinking substantially)

Go through the tubes one rack at a time. Pull out anything that looks like it needs transfer and put it in a separate rack. Fill up this rack with 20 tubes to be transferred, leaving two empty rows into which you put the new tubes. Then put on the labels for the new tubes, and keep the old and new tubes paired in this rack until the new culture has grown up. I usually allow one week for this before putting the new cultures away. I usually keep the old tubes for several more weeks, just in case.

Label the tubes BEFORE you transfer the cells. (This seems obvious, but I've had a couple of people working for me who wanted to transfer first and label later. That's dangerous). At Duke we used work-study students to put labels on tubes but the technician always did the actual transfers of cultures. Check to make sure labels match a) when you put the label on a new tube, b) when you transfer the cells from one to the other, and c) when you put the new tube back in the rack in place of the old one.

If a culture needs to be streaked on a plate for rehabilitation, make sure it is clearly labeled[2]. I recommend keeping a "hospital shelf" dedicated to such cultures, and a list of what's there at any given moment. This keeps them all in one place where they can be inspected daily, and mitigates against accidental loss.

[2] case history: we had an Israeli technician who made 1's that looked like American 7's. When she went on maternity leave, someone else took over some cultures she had on plates, but didn't read the numbers correctly, so a plate of strain CC-919 was put into a tube labeled CC-979.

Cleaning up contaminated cultures

If, despite your best efforts, a strain gets contaminated, here's a suggested triage system:

1) Check the other set of cultures; if there's a good one there, use it as a replacement and discard the contaminated one. Make a note of this in your files, just in case a problem arises in the future.

2) If both cultures are contaminated, proceed to cleanup efforts:

a) bacteria

For cultures heavily contaminated with bacteria, streak the culture on the most minimal medium on which the strain in question will grow; e.g., if it will grow on minimal, use HS or Tris-minimal; if it requires acetate, use HSA or TAP but omit the yeast extract. The idea is to enrich the Chlamy relative to the bacterial population before proceeding to the next step

If the contamination is slight, check under a dissecting scope to see if there are any uncontaminated areas, and try to recover a clean colony from there.

If there is slight but uniform contamination (Chlamy is still bright green, seems to outnumber the bacteria), then streak several plates of YA medium with it. Make one line across the plate with a wire loop or toothpick. Then, under a dissecting scope, use another sterile toothpick or glass needle to streak some Chlamy-rich areas perpendicular to this line, in hopes of getting a single colony that's bacteria-free.

Revisit this plate daily, looking for green colonies. Continue to use a glass needle to try to chase the Chlamy away from the bacteria. If it's apparent that the bacteria are overtaking the plate, you may need to use another tactic.

We prefer not to use antibiotics to clean up cultures. It's not an environmentally friendly practice, and often it doesn't work very well either. However, if the enrichment technique mentioned above isn't helping, sometimes a round on antibiotic medium will help and will at least buy you some time before the Chlamy are killed. Ampicillin is the best choice (doesn't reduce Chlamydomonas growth at all). Neomycin is another one that doesn't kill Chlamy but gets a lot of bacteria. Tetracycline is another possibility, but is inactivated by light so that's somewhat incompatible with use on a photosynthetic organisms. If you happen to be working with a Chlamy mutant that's already resistant to streptomycin, erythromycin etc., of course you can use that.

Another enrichment trick for Chlamy strains that can swim is to use phototaxis to concentrate them at the top of a liquid culture, then plate out cells from there.

Bottom line: patience, a dissecting scope and a glass needle usually works for bacterial cleanup. The other enrichment steps just facilitate your getting to the point where this is effective, and

rarely result in complete removal of the contaminant. Practice doing this before you need it to save a critically important strain.

Spray plating

For an irreplaceable strain, spray plating, as described in the Chlamydomonas Sourcebook, is often effective. Briefly, grow cells to exponential growth (for contaminated cultures, maybe 2 days) in minimal medium. Place the cells in a 1.5 ml eppendorf tube, and place into the tube a sterilized 25 microliter glass micropipet. Blow filtered building air of the end of the micropipet to create an aerosol, then pass agar plates, made with minimal media, through the “cloud” to collect microdroplets. With practice, you can insure that the droplets, that are small enough to contain either a Chlamydomonas or a bacterial cell, are spaced far enough apart on the plate that clean Chlamydomonas colonies can be subsequently isolated.

b) fungi

Fungal contaminants are actually easier to remove than bacteria, providing you catch them in time. However, many fungi seem to produce compounds that are toxic to Chlamy, so you need to work quickly.

You don't want to shed fungal spores over your usual transfer area, so do this first step at a lab bench in some other room where it won't contaminate anything else:

If the plate is heavily and totally contaminated, use a sterile toothpick to wipe away some of the fungus from above a green patch of Chlamy. If you're dealing with a plate that has some fungal colonies but not a solid mass, you can probably skip this step.

Use another toothpick to streak a Chlamy-rich suspension onto a fresh YA plate.

Leave this plate overnight. What you want is for the fungal spores that you inevitably transferred along with the Chlamy to germinate, but not to allow time for the fungus to sporulate again, so don't let it go for several days.

Using a glass needle under the dissecting scope, gently chase some Chlamy cells down the outgrowing hyphae and collect them into a pile.

With a fresh toothpick, pick up this pile and streak it on a fresh YA plate.

Usually this is all you need to do, but be sure to watch that last plate closely for a few days to make sure the fungus doesn't return.

c) mites

If you find a tube or plate contaminated with several different types of bacteria, especially if the contamination seems to follow trails across the agar, you probably have mites.

THIS IS AN EMERGENCY.

Once these things get established, they are nearly impossible to eradicate.

If you have a backup of the same strain, dispose of the contaminated one by autoclaving. If you don't have a backup, examine the plate or tube under a dissecting scope, pick off several of the cleanest looking Chlamy colonies you can find and put them on fresh plates. Make sure you don't transfer any mite eggs along with them. (These are somewhat elongate, smooth microscopic blobs). After you've done the transfer, look at the new plates under the scope and make sure there's nothing you don't want on them. Then autoclave the original culture. Keep the new plates under quarantine and look at them daily; otherwise, treat them as for bacterial contamination above.

Remove everything from the shelf that the mite-infested plate was on and clean it with hot soapy water, lysol, and anything else you may have on hand. Cover the clean shelf with foil.

Examine every tube or plate from this shelf under the dissecting scope to make sure you don't have any more mites. Usually if there's one, there will be more.

Continue to inspect these tubes and plates daily. If you have a real infestation, you will quickly become familiar with the life cycle. The eggs gradually develop some texture and then hatch into six-legged larvae. The larvae then undergo a metamorphosis into small eight-legged creatures (technically called "protonymphs"). These get bigger and go through two more stages to become adults. The whole process takes a couple of weeks.

The key preventative measure to precent mite contamination is to keep the caps on culture tubes screwed down tight. Enough gases penetrate a tightly capped tube to keep Chlamydomonas healthy, and mites are detered from traveling from tube to tube.

Some strains that need special attention

C. eugametos and *C. moewusii* are generally very hardy, but they tend to turn brown rather than bleach on aging. Sometimes this change isn't easy to spot in a dense culture, so pay special attention to these. Most of them are in the 1400s and 1500s in the stock list.

A few strains are inherently not dark green. I will flag these in the main list of cultures so that you don't inadvertently select for a suppressed phenotype.

The strains that are kept in dim light are primarily acetate-requiring, non-photosynthetic strains, although there are a few exceptions that don't grow in bright light for other reasons.

These tend to be finicky. They don't last long in tubes, and often don't achieve lush growth there. I find they do best on plates containing 2x the usual acetate concentration. Try to keep a stack of three plates of each one, representing successive transfers at intervals of maybe 3 to 4 weeks (or more often if needed).

Generally speaking, I don't like to put parafilm on petri plates, but in your drier climate it may be necessary to keep them from drying out. The main problem with it is that it can obscure your view of the Chlamy, so it's harder to see how healthy they are. Also, it's labor-intensive.

A few strains are so light-sensitive that they have to be kept in the dark. We've found the best system is to keep these as individual plates wrapped in foil. We maintain three sets of them, on different rotation schedules. Since there are only 24 of these strains currently, this isn't a big burden. Just set aside a regular time every couple of weeks to process one set of them. Try to keep a total of three transfers worth of plates in each set.

Be especially careful with the strains in this group that are nearly colorless, that you are really transferring Chlamy cells rather than perpetuating a bacterial or yeast contaminant.

A couple of the strains that should be nearly white have shown a tendency to drift toward green.

The y-y mutant (CC-2911) is supposed to be yellow whether grown in the light or dark. It is very prone to pick up suppressors and become green. This is less likely to happen if you keep it in dim light. If the stock you have now is yellow, keep it that way by being very careful when you transfer it, and subcloning for single colonies occasionally. The one I have here at Duke currently (February 2005) is green. We have had maybe ten different isolates of this thing over the years, and every one of them has eventually gotten suppressed. However, there are two points of good new on this one: first, the original phenotype can be recovered by outcrossing to wild type, and second, it's also been deposited in the American Type Culture Collection.

Left to right:

healthy culture
two tubes that are pale
two tubes starting to bleach from the top and turn olive
healthy culture but with agar shrinking due to dehydration