



Title: *The Basics of Hybridoma Cell Culture(from Start to Freeze)*

No: RTLP-GL-Ab-5

Location:
Old CCRC Tripp Lab

Approval Date:
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Supersedes Date:

Materials:

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|----------------------------------|-------------------------------------|---------------------------|-------------------|
| •Lab coat | • β -Mercaptoethanol (BME) | •24 or 48 well plate | •Pipettes |
| •Gloves | •RPMI 1640 | •50 mL tubes | •Pipetteman |
| •Media: RTLP-GL MP1-8 | •37°C, 5% CO ₂ incubator | •15 mL tubes | •Pipette Aid |
| • Hybridoma Cloning Factor (HCF) | •Sodium pyruvate | •Centrifuge | •Pipetteman tips |
| •37°C H ₂ O bath | •Centrifuge | •1, 12, & 60 mL syringes | •18 gauge needles |
| •Tissue culture flasks | | •1 mL syringe | •Transfer pipets |
| •cryovials | | •2- 0.2mm syringe filters | |

- Thoroughly clean tissue culture hood and incubator at least once a month.
- We generally grow the cells in RPMI 1640 medium supplemented with fetal bovine serum (typically 10%) and Origen Hybridoma Cloning Factor (HCF) (to 5%), with Pen-Strep, extra glutamine, and sodium pyruvate.
- When we are thawing cells that have never been subcloned (these have names like X2C11) we also need to add some HT (hypoxanthine, thymidine) since the cells may still be stressed from the aminopterin present when we first made the hybridomas back in 1992. Also, when thawing these cells, it may be helpful to have some beta-mercapto-ethanol in the medium.

Additional Medium:

WASHING MEDIUM:

- 100 ml newly opened RPMI1640.
- 10 ml FBS (regular IgG levels-perhaps IgG acts as a cytokine for these cells)

- 50 µl of diluted beta mercaptoethanol (dilute 5µl to 720 µl distilled water to get 0.1M-concentrated BME is 14.3 M-if we then take 50µl to 100ml we get 0.05 mM final concentration)
- 1 ml L-glutamine (Fisher MT-20-005-LI)
- 1 ml Na-Pyruvate (Fisher MT-20-000-LI from 6x100ml bottles)
- 1 ml Pen Strep (10,000U/ml penicillin + 10,000µg/ml streptomycin)
- 100µl 10mg/ml gentamycin (Sigma G-1275) - optional, routinely we add PenStrep.
- Add 1ml of 100x HT for cells that have not been subcloned.

PLATING MEDIUM:

To 15 ml of the above add 9 ml of FBS and 6 ml of Origen hybridoma cloning factor (HCF). Filter sterilize. This gives us 30 mls of rich-medium in RPMI.

If we wash the cells in 8ml of 10% FBS in RMPI (plus other stuff in 'wash').

Procedure:

NON-SUBCLONED:

1. Thaw cells into medium with HT & BME and into 48 well plate
2. Transfer to 24-well plate and set up subcloning on 96-well plate
3. Transfer to T25 (take sample supe for IF & ELISA)
4. Refreeze from T25

FROM SUBCLONED PLATE:

1. Score plate
2. Transfer to 24-well plate (take sample supe for IF & ELISA)
3. Transfer to T25
4. Freeze from T25

In either case, keep good records, & coordinate with ELISA and IF work & transfer to liquid nitrogen & record-keeping.

ALREADY SUBCLONED:

1. Thaw cells onto 24- or 48-well plates.
2. Transfer to T25 (take sample for IF)
3. Refreeze from T25. (keep good records)
4. Grow for supernatants (since presumably that is why we are thawing).

GROWING FOR SUPES:

1. Split a single T25 into 3 T25's and do this until you have 6-8 T25's with your clone.
2. When cells are dense, "knock them back" by hitting the flask, transferring about 3/4's of the cells and medium to a T75, and refeeding with medium (typically dropping from 10:5 to lower FBS or to a reduced serum medium).
3. Let the cells in the T75 slowly die. While alive, they will secrete antibody into the medium.
4. Harvest the supernatant, add thimerosal to 0.01% as a preservative.

TRANSFERRING CELLS-24 well to T25:

When the cells become dense enough (this is probably best learned experientially, the minimum time will likely be 2 days, the maximum time so far has been about 2 weeks), they need to be transferred from the 24 or 48-well plate to a T25 flask. Transfer by pipetting up and down to dislodge the cells from the plate, then transfer them to a new T25 flask containing medium that is at least 10% FBS; 5% HCF.

Typically, if the cells were healthy going from 24-well to T25, then in 2-3 days they will be ready to freeze. See that section.

For **routinely growing cells for supernatants (to collect antibodies)**: gradually lower the amount of FBS and Origen

hybridoma cloning factor in the medium. So, when you've refrozen the cells from the T25's, instead of adding 15%FBS/10% Origen hybridoma cloning factor to the remaining cells in the T25, refeed the cells with 10% FBS/5% Origen hybridoma cloning factor. Then next time you refeed (after putting most of the cells and supe into a T75) use 10% FBS/2% Origen hybridoma cloning factor. Thereafter drop to 8%/2%, then 5%/2%. I haven't had much luck growing the cells lower than that-some clones adjust well, others don't. To drop lower than 5%/2% you will have to test each clone individually. There are some FBS-replacements on the market and these may work for your clone of interest, but each has to be tested individually.

What I have found to work quite well for collecting supernatants for purifying antibodies is to grow the cells in the T25 flasks and split them leaving 1/8th or 1/10th in the T25 and putting the remainder into a T75 and just let the cells die slowly in the T75 (that'll take 2-4 weeks, all the while they'll be secreting antibody, we hope). The cells growing in the T25 flasks will start to look abnormal after 3-4 weeks since we are selecting for ones which don't detach easily.

Another option is to switch to a serum-replacement medium like HyQ-CCM. This is more expensive than RPMI but we will not need to add much, if any, serum. So, purification of the antibodies is less of a problem.

Typically, to make the switch without shocking the cells, first have the cells in normal

medium, then to a mix of 25% HyQ-CCM and 75% normal, then 50:50, then 75:25, then finally to HyQ with a little Origen hybridoma cloning factor. You will need to add antibiotics to the HyQ-CCM medium-1ml of the 100x PenStrep for each 100ml. Also, add 5ml of Origen hybridoma cloning factor (this may not be necessary if the cells grow very well).

THAWING

Thawing:

Cells are stored in liquid nitrogen in cryotubes. Use caution when removing vials from liquid nitrogen, only do when someone else is present with you. (This is hazardous since the tubes can explode or other problems may arise.) Wear the face mask and full clothing (including lab coat; long pants; full shoes). The cells have been frozen in a medium containing DMSO (dimethylsulfoxide). This is toxic to the cells at room temperature, so be well organized and thaw the cells quickly and get them diluted into medium containing FBS as quickly as reasonably possible.

Getting set up:

A. Know what cells you are going to thaw and move vials from liquid nitrogen to either -70C (for future thawing-either put in box or in transfer vessel) or to an isopropanol-containing transfer vessel (if thawing within a few minutes). Update records accordingly.

B. Get things ready for thawing.

- Thoroughly clean tissue culture hood and incubator.
- Prepare Washing and Plating Medium (see below) from a fresh bottle of RPMI. For cells frozen back in 1992 and shipped from Kansas, use 30% FBS; 10% Origen hybridoma cloning factor. If a particular hybridoma doesn't thaw well in this, then try even richer if necessary--go up to 50% FBS; 15% Origen.
- Have 8mls of Washing Medium - see below- in 15ml centrifuge tube ready for the cells.
- Have centrifuge reserved and know it is working.
- Have the 24-well plate ready and the Plating Medium handy.
- Really wash hood well before making the medium and also just before thawing the cells. (In some cases we only have one vial of a hybridoma.)

C. Actually thawing:

1. Make 37C water bath in tissue culture room (can use water heated in microwave, and then put into a styrofoam container).
2. Place vial into chilled (-70C) transfer vessel and bring to tissue culture room.

3. Thaw vial of cells quickly at 37C in water bath. Decontaminate outside with ethanol and put in hood. Remove contents quickly and disperse well in the 8ml of Medium in the 15 ml tube.
4. Spin the 15ml tube (with cells) 100g 10min and discard supe. (This step dilutes the DMSO to non-toxic levels for the cell.)
5. Put approximately 1ml plating medium into each of the three wells of the 24-well plate.
6. Resuspend in about 1 ml of plating medium and put about 1/8 in one well, 1/4 in another; 5/8 in another of 48-well plate. Add more plating medium to even up the volumes per well. This allows different concentrations of cells/well which in some cases is beneficial.
7. Place in CO₂ incubator and leave undisturbed for 12-24 hours for cells to attach and equilibrate. The cells are sensitive at this time so do not stress them by leaving them out of the incubator.
8. Observe them carefully over the next few days. One common 'fault' is to care too much for the cells and change the medium too often. The cells need cytokines to grow well and they produce and secrete some of these cytokines-give them a chance to build up the concentration of cytokines in their medium.

For **thawing cells**: For thawing the original hybridomas (not the subclones) make sure you add HT since the hybridomas were frozen while still in HAT medium. Also, some of the original clones were frozen under poor conditions. I routinely open a FRESH bottle of medium JUST PRIOR to thawing. Make about 10 mls for plating the thawed cells: 20% FBS and 10% Origen hybridoma cloning factor. If a particular clone doesn't grow even in that rich medium, consider going to 30% FBS and 15% Origen hybridoma cloning factor. Most of the subcloned lines probably would thaw fine in 15% FBS; 10% Origen hybridoma cloning factor and maybe even lower. I strongly suggest having some 100ml bottles of medium that you open the day you thaw from liquid nitrogen. Although cells MAY grow in other conditions, they'll probably grow better (saving you time, worry, energy, resources) at a minimum investment.

SUBCLONING CELLS:

If the hybridoma has a name like X17E9 (letter X or Z, then number, then letter, then number) it hasn't been subcloned. Subcloning as we do it by limiting dilution, means we dilute the cells to reach a dilution in which only one cell is present in a well and that cell divides and forms a clone of cells. Hence the name, **monoclonal**. To do this we need to have a rich medium, since the cells don't like being so alone. They need growth factors not present in the medium but that they themselves will secrete for themselves and each other. The more cells, the quicker they secrete the amount of growth factors they feel happiest in. A cell by itself has a tough time. Yet, that's what we are asking-for a single cell to divide and form a clone. So, we provide a rich medium and good growth conditions.

As a first try, use 20-30% FBS and 10-15% HCF for the medium. Set up the dilution as follows: (this is what works reasonably well for me)

1. Thoroughly clean tissue culture hood and incubator.
2. Make the medium and put 100 μ l into each well of one-half of a 96-well plate.
3. From a very well growing 24-well or T25, take one drop (50 μ l) of dispersed cells into 100 μ l medium of the first well of the 96-well plate half you are using.
4. Dilute this serially across the top row, taking 50 μ l to the next well, dispersing it well, taking 50 μ l of that to the next well, etc. (so 1:3 serial dilutions across the plate).
5. Check under the microscope and guess which 3 wells will contain approximately the right number of cells so that when you dilute **DOWN** the plate, at some point you will have **NO** cells in the wells.
6. Choose those wells and dilute **DOWN** the plate by taking 100 μ l from those wells and sequentially diluting down the plate (so 1:2 serial dilutions down the plate). Take the 100 μ l from the bottom row and put it back into the starting wells.
7. Repeat from the same starting wells (which are now diluted 1:3 from what they were at the start) and repeat.
8. Place the 96-well plate into the incubator and treat with care-you want the cells to be undisturbed when they divide so each cell forms a tight colony that you can easily see. If you bang the plate then what would have been a single clone will appear as multiple clones and you are ___ out of luck and will need to start again.
9. Leave the plate alone for 3-4 days, undisturbed. Then 'score' the plate by looking at each well and marking which wells contain only a single clone. It helps to also mark which wells contain **NO** cells/clones and which diluted wells contain multiple clones.
10. Leave the plate another 1-2 days, and rescore.
11. When the cells have reached an appropriate size (filling perhaps 1/6th of the field at low power), transfer the individual cells from the 96-well plate to a 24-well plate. Transfer by pipetting up and down to disperse the clone then transferring most of the cells to a 24-well plate containing about 1/2 ml of rich medium (at least 10%:5% FBS:HCF, but could be higher). Label everything well through this process. If we are assigning a subclone number (and I expect we would be) then make sure you document what number is assigned to what clone.
12. At appropriate time (cells covering perhaps 1/8 to 1/4 of the surface area of the 24-well) transfer to T25 as discussed in that section. After growing in T25 you will likely have to freeze the cells (so we have stocks in the freezer).

For **subcloning** of the hybridomas: add 10% Origen hybridoma cloning factor (the Origen hybridoma cloning factor provides growth factors - previous protocols called for feeder cell layers) and increase the FBS up to 20%.

FREEZING CELLS:

We freeze cells so we have a stock of them for the future. Whenever we subclone, we will need to freeze. At other times, we may also have to freeze. Basically, for freezing we are going to treat the cells with a medium that contains DMSO which is lethal to the cells at room temperature if they sit in it for long. So, be organized and aim at having the cells in Freeze Medium (Origen Freezing Medium, from Fisher) for no longer than 15 minutes at room temperature. By organizing things in advance, this is feasible.

You will need:

- freeze medium (this cannot be filter sterilized with the filters we have)
- cryotubes
- marker for cryotubes
- notes for record-keeping
- low-speed centrifuge for pelleting cells
- 15 ml tubes
- transfer pipettes
- more medium for refeeding cells (typically 10%:5% FBS:HCF)
- transfer vessel (contains isopropanol so cells are chilled at 1C/min rate to -70C temperatures
- if you remember, have the transfer vessel prechilled to 4C before freezing cells)
- You also need VERY HEALTHY cells-we want the cells to survive being chilled to, and thawed from, liquid nitrogen temperatures. This is harsh on cells (small wonder) and we want to recover them later! So, make sure what you are freezing is healthy, if at all possible. You want the cells growing logarithmically. If there is any chance they are overgrown (even if they aren't yet looking too bad), then it is best to remove the supernatant and loose cells and refeed and then freeze the next day.

TO FREEZE:

1. Have cells in T25 flasks, occupying perhaps 1/4 of the space for the bottom 1/4 of the T25 or else forming a dense layer across the very bottom of the flask. Get everything else organized, including your notes for labeling tubes (and perhaps have the tubes already labeled as well).
2. Hit the flask to dislodge the cells, transfer the cells and medium to a new 15ml tube (well-labeled) and spin 10min 100g. (While spinning, label cryotubes if not already done). Add new medium to the T25 flasks to let the remaining cells grow and give us some more supernatant for the future.
3. Remove supe from cells, retaining it into a new 15ml tube (also well-labeled).
4. Resuspend cells in 1.5-2ml of Freeze Medium and aliquot ~0.5 ml (10-14 drops from the transfer pipette) into the cryotubes. Cap the cryotubes tightly.
5. Immediately get the cryotubes into the transfer vessel, preferably prechilled to 4C, and into -70C.
6. Finish clearing the hood, etc.

Author	Management Approval/Date	Effective Date

7.