Bacterial Production (RADs) Protocol

Modified September 2019
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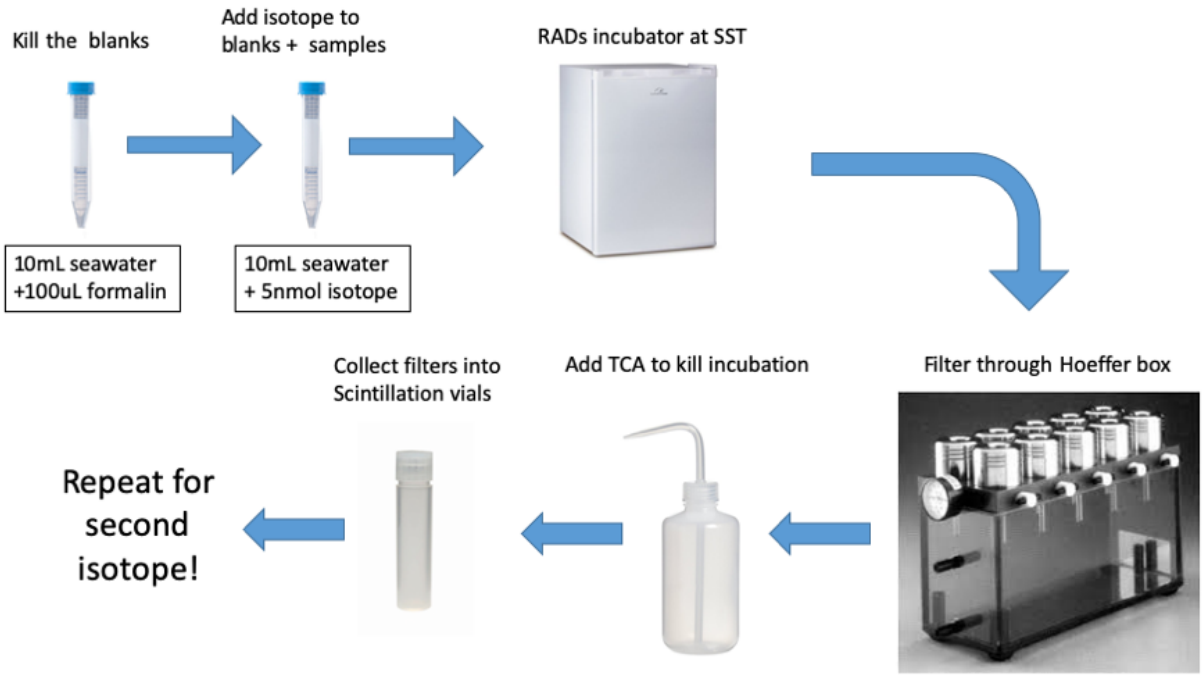
I. Visual overview of protocol:



**Disclaimer: Tritiated Leucine and Thymidine are beta emitters, which is a fairly safe form of radiation. It is incapable of penetrating through clothing/ gloves, but dangerous if ingested. We go to extreme lengths to prevent contamination in the lab: treat everything you touch while doing this protocol as though it were radioactive. Always wear PPE and wash your hands often!

II. Prior to Starting

- Double check that the RADs incubator is set to the correct sea surface temperature (SST). Confirm the SST with someone who was aboard the ship.
- Ensure that the filtration towers and the TCA have chilled in the RADs refrigerator overnight. Double-check that you have at least 500mL of 5% TCA.
- Double check that you have enough 0.45µm HA Millipore filters, and sufficient volume of isotopes.
- Take care of whatever YOU need in terms of food, water, bathroom, etc. The next part of this protocol usually takes me three hours.

III. Hands-on Protocol: Complete on SPOT Night

1. **Collect and arrange the samples**
   - Ensure that all subsamples are properly labelled on the tops and sides (especially blanks!), and have been rinsed 3x and filled to 10mL with seawater.
   - Arrange them on the bench above the RADs incubator: separate Leucine samples from Thymidine samples.

2. **Kill the blanks** by adding 100uL (0.1mL) of 0.02µm filtered formalin.
   - Only take the blanks over to the chemical hood. Do not wear radioactive coat/ gloves yet, because this PPE should not be taken out of the RADs area.
   - You can fill out the top part of the RADs data worksheet at this time (include your name, the date, and the RCM (identification number handwritten on the top of the isotope), volume, and specific activity of the isotopes you are using). Ideally, double check the calculated volume of isotope to use (see appendix).

2. **Add 5nM Leucine** to all of the Leucine samples, including the blanks.
   - Unless you are using a new aliquot of isotope, add the same volume of isotope that was used the month prior to your experiments.
   - Try to add the isotope to each sample at the same time; work as quickly (but safely!) as possible. To avoid droplets when pipetting, dispense the isotope with moderate force, keeping the tip inside the lip of the tubes. It helps to loosen all the caps of the Falcon tubes and set the pipet to the correct volume *before* you get out the isotope.
   - RECORD THE TIME that you add the Leucine.
   - Store the samples in the RADs incubator for one hour. Ensure that the surface samples stay close to the light bulb for the entire incubation. Place the DCM samples in the rack with 2 layers of mesh in the incubator door. Place the samples from deeper depths in the cardboard box, shielded from light.
   - Note that the procedure for working with Leucine and Thymidine samples is very similar, but we offset processing these samples by 30 mins.

   Helpful tips for working with isotopes:
   - You should be wearing radioactive PPE from this point forward.
   - At this point, the Falcon tubes are arranged in two plastic tube racks on the bench behind the RADs area. Do not take the plastic tube rack into the radioactive hood: anything that touches the hood must stay there. We have plenty of tube racks in the hood! Instead, only bring the Falcon tubes into the hood; lift them out of the rack.
   - Arrange the samples in a radioactive plastic so that you remember where the blanks are! I prefer to use the 3+1 pattern: arrange the three replicates in an upside down L shape and put the blank in the corner, as follows:

Sample	Sample
Sample	**Blank**

   - Minimize your exposure to the isotope. Keep the Leucine/ Thymidine out for as brief a time as possible.
   - It is okay to start with Thymidine (i.e. switch steps 2 and 4), but I prefer to do Leucine samples first because there are more of them.

Helpful tips on timing/ workflow:

- At this point, I start three timers. One for 30 minutes (signaling when to add the Thymidine), one for 45 minutes (signaling when to set up the Hoeffer boxes), and one for an hour (signaling the end of the Leucine incubation).

3. **Label 45 scintillation vial lids** and document those numbers in the RADs binder.
   - Do not touch the lip of the vial or the inside of the cap with your hands. Grasp the bottom of the vial and fit the lip into the cap without touching the cap.
   - Double-check the previous page of the RADs binder to ensure that you are starting on the correct number. Vials are labelled consecutively from month to month.
   - Label the tops of the lids only; do not write on the sides of the scint. vials or lids.
   - The blank is always the last sample in each group of four.
   - There should be 32 scintillation vials for the samples (20 Leucine samples + 12 Thymidine samples) and 13 vials for the wipe test (letters A-M – 11 surfaces + 2 blanks). (Note that we only use Thymidine for the top 3 depths.)

4. After Leucine samples have been in the incubator for 30 minutes, **add Thymidine to all of the Thymidine samples and store in the RADs incubator for one hour.**
   - See "Helpful tips for working with isotopes," above (#2).
   - As above, ensure that the surface samples stay close to the light bulb. Place the DCM samples in the rack with 2 layers of mesh in the incubator door. Place the samples from deeper depths in the cardboard box, shielded from light.

5. 15 minutes before the Leucine incubation ends, **set up the Hoeffer box and towers.**
   - Rinse the filter box cover and tweezers 3x with DI water, and fill the box with water.
   - Throw in filters to help separate the filters (white) from the spacer paper (blue).
   - Place the wet filters in the grooves of the Hoeffer box.
   - Get out the chilled towers, and place on top of the filters. Push the towers down and twist to ensure an adequate seal.
   - Close all the taps!

6. **Filter the Leucine samples through** HA filters at <0.3atm.
   - The Thymidine samples still need to incubate for 30 minutes. I'd reset your 30 minute timer here.
   - Pour all the samples into the towers, THEN open the taps, THEN turn on the pump.
   - Keep track of where the blanks are!
   - Try to pour at a steady rate so that no droplets are spilled. Try to get the last drops out of the tip of the Falcon tubes.
   - If you spill any droplets of sample, or if a sample leaks out from under the tower, document it in the RADs binder. If liquid leaks out under the tower, wipe it up with Kimwipes to avoid cross-contamination.
   - Check that the sample is actually filtering through; liquid should be dripping from the taps into the Hoeffer box at a quick rate. If the sample is not passing through, loosen

the valve. If none of the samples are passing through, you do not have an adequate seal on the Hoeffer box. Try tightening all the valves.
- When it looks like all of the liquid has passed through, turn off the pump and close the taps.

7. **Add TCA to kill the incubation.** Squirt cold 5% TCA around the inside of the filter 3x slowly – this should give you a volume of ~2mL TCA.
   - Count to yourself as you rotate around the tower (1-apple, 2-apple, 3-apple).
   - If you have spilled any droplets of samples on the rim of the towers, try to wash them in with the TCA.
   - RECORD THE TIME when you add the TCA. Calculate the incubation time (from when the isotope was added until the TCA was added).
   - Let the filters incubate in TCA for 2 minutes.
   - Open the taps again, and filter through the TCA.

8. With the pump still on, **rinse the towers 3x with TCA.**
   - This time, rinse around each tower once, then twice, then a third time.

9. **Remove the towers** and **rinse the exposed filters 3x with TCA.**
   - The pump should still be on!
   - If any liquid spills out from under the towers as you remove them, wipe it up with Kimwipes to avoid cross-contamination. Avoid touching the filters.
   - Make sure the filter gets completely covered with TCA in each rinse.

10. Turn off the pump, remove the filters, and **place filters into scintillation vials**.
    - Be careful not to tear filters; they are fragile.
    - The sample side of the filter should face the inside of the vials.
    - Do not touch the forceps to the middle of the filter; handle filters by the edges.

11. Quickly **rinse the towers and the tops of the Hoeffer boxes** 3x with DI water.

Repeat steps 5-10 with the Thymidine samples, as follows:

12. **Set up the Hoeffer box and towers**, as in Step 5.

13. **Filter the Thymidine samples through** HA filters at <0.3atm**,** as in Step 6.
    - Note that you do not need to use all the wells for the Thymidine samples (in contrast to Leucine samples). Be sure to tighten the taps on any wells you are not using.

14. **Add TCA to kill the incubation,** as in Step 7.

15. **Rinse the towers 3x with TCA,** as in Step 8.

16. **Rinse the filters 3x with TCA,** as in Step 9.

17. **Place the filters into scintillation vials.** You're almost done for the night, just clean up!

III. Clean-up Protocol: Complete immediately after Part II on SPOT Night

1. Carefully pour the radioactive filtrate out of the Hoeffer boxes and into the liquid waste container. Use the funnel.

2. Rinse the following 3x with DI water: Hoeffer boxes, the tops of the Hoeffer boxes, the filtration towers, the box used to separate out filters, the tweezers. Set the towers and Hoeffer boxes and tops inside the RADs hood to dry overnight.

3. Spray areas A-K liberally with NoCount, and wipe them down.
   - Change your gloves before you start cleaning up.
   - Shake the NoCount before spraying.
   - Try to let NoCount sit on the surface for a few minutes before wiping it up.
   - Change paper towels for each new surface.
     A. Hood counter (also open up the hood and clean beneath it)
     B. Floor under the hood (this includes the surface of the acid storage cabinets under the hood; be especially thorough with the handles)
     C. Outside of the liquid waste container
     D. Floor below the liquid waste container (including the edge of the brown secondary container)
     E. Dry waste container
     F. Rad work counter top (usually covered with paper, so just spray around the sink)
     G. Floor below the rad work counter top and fridge (again, this includes the cabinet surface and handles)
     H. Inside the sink
     I. Floor below the sink
     J. RADs fridge door
     K. RADs incubator door
     L. Lab door handle leading to the Heidelberg lab
     M. BLANK

4. Complete a wipe test for these surfaces.
   - Tear up little pieces of paper towel into 2cmx2cm squares and soak them in DI water.
   - Using the tweezers, wipe down each surface with one towel piece, and place the it in the corresponding scintillation vial.
   - You should discard the towel pieces, change DI water, and rinse the tweezers every 3-4 letters.
   - Note that L and M are blanks! Do not use NoCount on the door to the Heidelberg lab, and for M, just put a wet piece of paper towel directly into a scintillation vial.

5. Add 5mL scintillation fluid to each scintillation vial using the fixed-volume dispenser on the RADs bench. Tightly cap the vials and shake them HARD.
   - This will create a fine emulsion (<1μm), which allows the hydrophilic beta particles to enter the hydrophobic scintillation fluid, enabling detection by the scintillation

counter. Beta particles can only travel ~1μm before they are absorbed; the emulsion is necessary for detection.

6. Store the scintillation vials in the dark for at least 3 hours, preferably overnight, using the cabinet under the RADs bench.

7. Throw any dry waste in the radioactive waste barrel, not the regular trash.

IV. Hands on protocol: Complete the day after SPOT

1. Run the samples the Beckman scintillation counter in the Manahan lab.
   - You need to wear radioactive PPE when carrying the samples, but you cannot touch a radioactive glove to any non-RADs surfaces. It's best to take one glove off to open doors etc.
   - Double-check with the Manahan lab that no one needs the scintillation counter urgently – our run takes a few hours. If someone else is running samples, you can load your samples into the machine without disrupting their run.
   - Get the small blue racks from the cart against the back wall. Each rack holds 18 samples. Arrange three racks so that the numbers are facing you.
   - Insert user card 01 into the slot on the back right of the first rack. The cards are kept in a drawer beneath the scintillation counter.
   - Shake the samples briefly (5 seconds) before placing them in the racks in order.
   - Leave a blank spot between the samples and the wipe test – this makes it easy to identify which values correspond to the wipe test.
   - Insert the racks into the scintillation counter, and be sure to fit the little tab into the groove of the machine. The numbers should face you as you load the racks; the machine will read left-right, like we do. Place a calibration rack (blue rack with two bottles, which are Thymidine and Leucine standards) before your samples and a halt rack (red) after.
   - Hit "Start" on the machine twice. You don't need to change any settings! The instrument is set to detect tritium, stopping each count at 5 minutes or 500 counts, whichever comes first.
   - You should stick around until the machine finishes calibrating and begins counting your samples.

2. Finish cleaning up by placing the filtration towers back into the RADs fridge to chill.

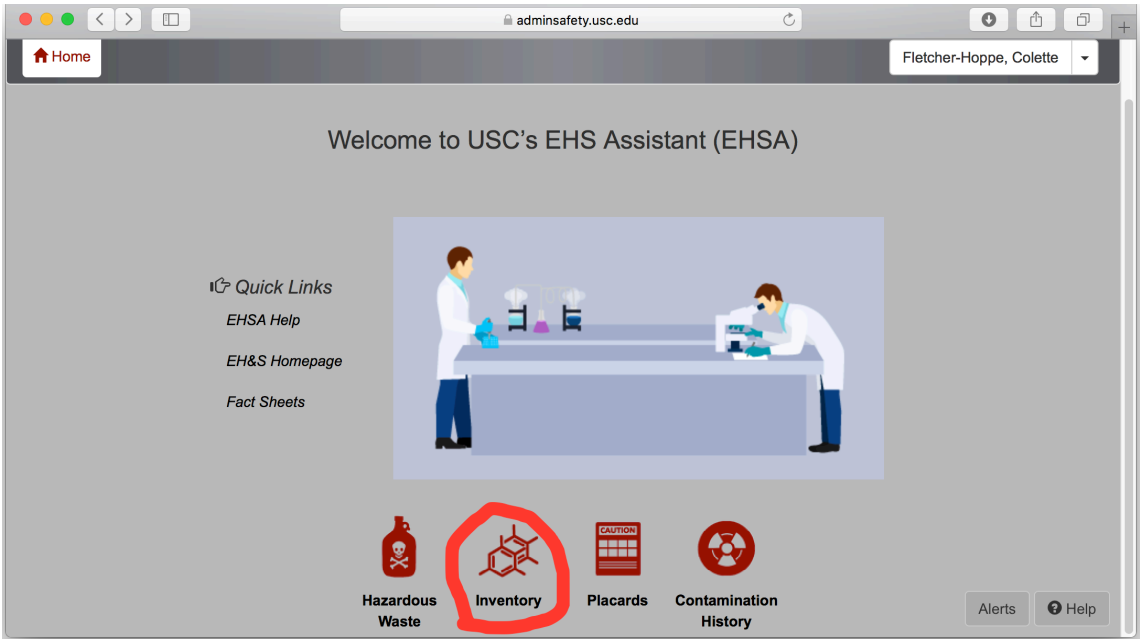
3. Double check that you have enough TCA for next SPOT. If not, dilute more.

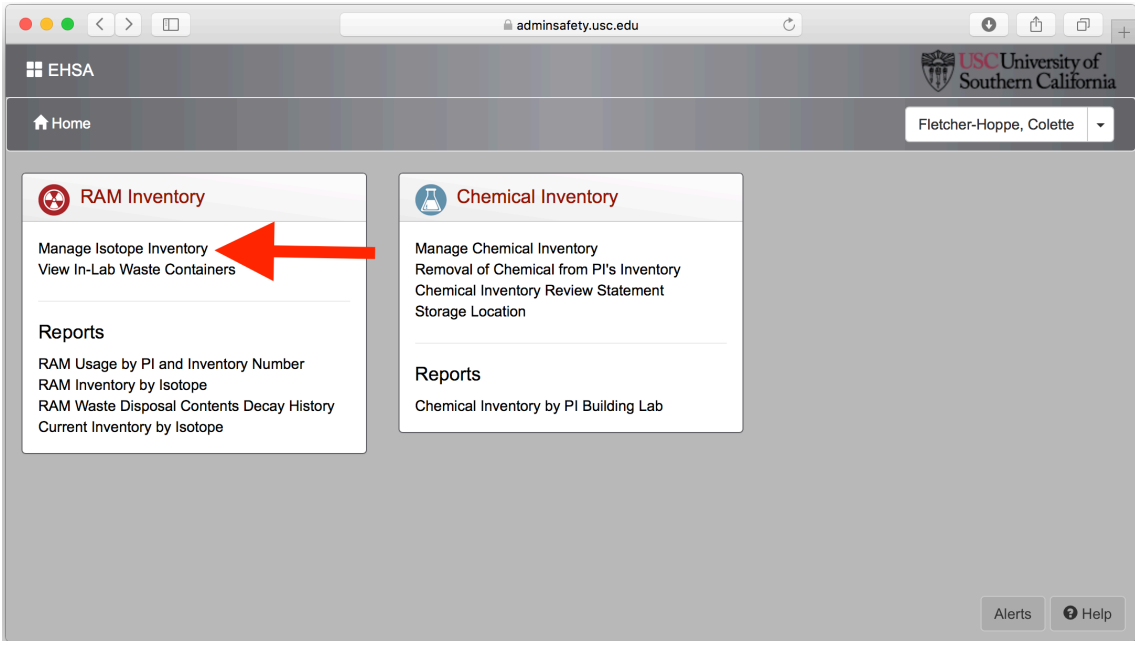
4. When your samples finish running, store the sample scintillation vials in a plastic bag in the cabinet under the RADs bench. Discard the wipe test vials in the metal drum closest to the RADs hood (which should only be full of wipe test vials).
   - Double check to make sure the lids are tightly capped to avoid leaks.
   - If anything goes wrong, you can re-run the samples, no problem. It is okay to run the samples multiple times and for up to three months after you did the protocol.

V. Computer-based protocol: Complete as soon as possible after running the samples

1. Record the RADs data on Dropbox.
   - The spreadsheet is called "Isotope_SPOT_1222011-3-2" and dates back to 2000.
   - Write down the SST, DCM depth, and incubation times for Leucine and Thymidine on the top of the datasheet. Make a note of which data correspond to which samples (e.g. the first four should be Leucine samples from 5m depth; #4 should be the blank for this group).
   - Double check the RMC number and specific activity of the isotopes.
   - Just type in the DPM for your three replicate samples and the blank. Double-check you got all the digits correct! Drag down the Excel file formulae to fill in the rest of the sheet.
   - Store the data in a page protector in the black RADs binder in Liv and Cesar's office.

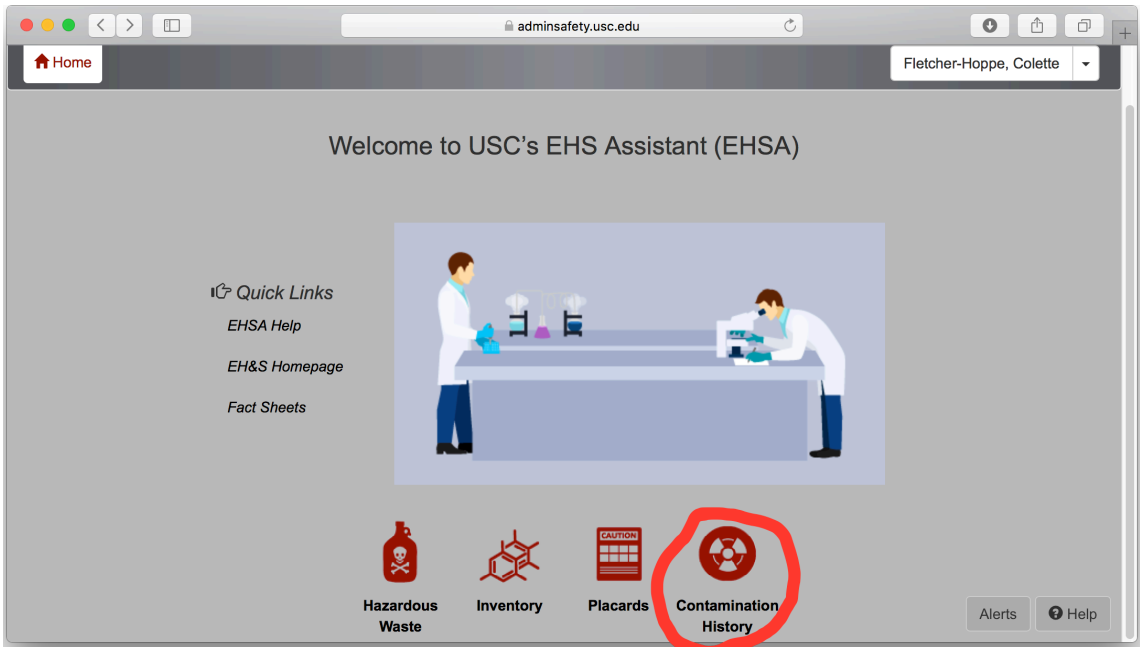
2. Update the RADs usage on EHS Assistant.
   **DISCLAIMER: these instructions are current as of 9/16/2019. The EHS site has been modified often, so the following instructions might be totally invalid in 6 months. **
   - Log into EHSA here: https://ehs.usc.edu/research/manage/ehsa/ (scroll down to the bottom of the page). If you are not able to log on, it means that Jed has not registered you as an approved user of the radioisotopes. Either ask him to register you, or have another student log in for you and you can use their account.
   - Click on the "Inventory" button, then "Manage Isotope Inventory" on the left.





- Double click on one of the isotopes (remember which RMC # is which!). Scroll down, and hit the "Add" button. Fill out the corresponding information, and double-check that the date of your entry corresponds to the most recent SPOT.
- For the volume used, multiply the volume of Leucine that you added to each sample by 20, and multiply the volume of Thymidine you added by 4 (4=12/3: we have 12 Th samples and we dilute Thymidine by 1/3 in ethanol).
- We have three categories of waste: 89% of the waste is liquid, 1% is solid waste, and the other 10% is liquid scintillation. For each category, select the appropriate waste container from the dropdown menu (there should be one container per category).
- Remember to do this for both isotopes!

3. Update the Contamination History, and upload the wipe test results.
   - Go back to the EHSA home page, and hit "Contamination History" at the bottom of the page.



- Hit the "Add" button at the top of the page, and fill in the appropriate information.
- Under "Detector used," select the Beckman scintillation counter.
- "Locations" should be AHF 231E.
- Did you find any contamination? If any of the wipe test results were EITHER greater than 100 DPM OR greater than 3x the value of the blank, then yes, you do have contamination. You will need to re-do the wipe test. Only upload documentation for the wipe test that does not indicate contamination.
- Under "Documentation," upload a .PDF of the wipe test. You will need to do this from the computer in the breakroom.
    - Open the scanner from the computer by double-clicking "HP OfficeJetPro 8710" icon on the desktop. Select the computer connected via USB.
    - Hit "Scan," "Save as .PDF," then scan it. You can adjust the contrast and crop the image to make the text more readable.
    - Don't forget where you save the file. Upload it.

That's it! You're done! ☺

VI. Appendix

- Ordering new isotopes:

Item	Cat#	Price	vendor
Leucine, L-[3,4,5-3H(N)]-Leucine, Specific Activity: 100 to 150Ci/mmol (3.7 to 5.56 TBq/mmol), 5mCi (185MBq)	NET460005MC	$736.00	Perkin-Elmer
Thymidine, Thymidine, [Methyl-3H]-, Specific Activity: 70-90Ci(2.59-3.33TBq)/mMole, >97%, 1mCi (37MBq)	NET027Z001MC	$545.00	Perkin-Elmer

- Once the new isotopes have arrived, immediately dilute the Thymidine by 1/3 in molecular grade ethanol (e.g. add 2 mL EtOH to 1mL Th). Aliquot 1mL of this dilution into 3 1.5mL Eppendorf tubes.
- Immediately store the Leucine in the RADs fridge door.
- Calculate the volume of isotope you need to add to the samples (remember you should add 5nM per sample, or 5nmol/L).
    1. Activity of the isotope will be given in Ci/ mmol on the label. Concentration is 1 mCi/ mL.
    2. Get the concentration into moles/ mL $(\frac{1}{activity\ (\frac{Ci}{mmol})} * \frac{1\ mCi}{1\ mL})$
    3. Multiply by 1000 to convert the concentration to moles/L.
    4. Use $C_1\ V_1 = C_2\ V_2$ to calculate the volume needed for 5 nmoles in 10mL of sample.
    5. If you are using Thymidine, multiply this number by 3 to account for the 1/3 dilution in Ethanol.
- Examples: try to calculate the volume on your own and see if you get the same answer.
    1. Leucine at 100 Ci/ mmol → Add 5µL/ sample
    2. Leucine at 150 Ci/ mmol → Add 7.5 µL/ sample
    3. Thymidine at 80 Ci/ mmol → Add 12µL/ sample
- If you get the math wrong and use the wrong volume of isotope, don't adjust the DPM. The important thing is to saturate the samples with isotope, and 3nM usually does the trick. So calculate for 5nM and don't worry if you're a little off.

- Requesting waste pick-up: Do this when the container is ¾ full
    - Log onto EHSA. Click on "Inventory," as above.
    - Click "View in-lab waste containers" (second link in the left column)
    - Click "Request Pickup" on the appropriate container. Also "Seal" the container – this means you agree not to open it or discard additional waste there.
    - Fill in the required fields.
    - Print this page and attach it to the container you need to have picked up.

Summary of hands-on protocol:

I. Prior to Starting

- Ensure that all subsamples are properly labelled on the tops and sides (especially blanks!), and have been rinsed 3x and filled to 10mL with seawater. Arrange them on the bench above the RADs incubator: separate leucine samples from thymidine samples.
- Double check that the RADs incubator is set to the correct sea surface temperature (SST). Confirm the SST with someone who was aboard the ship.
- Ensure that the filtration towers and the TCA have chilled in the RADs refrigerator overnight. Double-check that you have at least 500mL of 5% TCA.
- Take care of whatever YOU need in terms of food, water, bathroom, etc. The next part of this protocol usually takes me three hours.

II. Hands-on Protocol: Complete on SPOT Night

1. **Kill the blanks** by adding 100uL (0.1mL) of 0.02um filtered formalin.
2. **Add 5nM Leucine** to all of the Leucine samples, including the blanks.
3. **Label 45 scintillation vial lids** and document those numbers in the RADs binder.
4. After Leucine samples have been incubating for 30 minutes, **add 5nM Thymidine to all of the Thymidine samples and move the Leucine samples back.**
5. 15 minutes before the Leucine incubation ends, **set up the Hoeffer box and towers.**
6. **Filter the Leucine samples through** HA filters at <0.3atm.
7. **Add TCA to kill the incubation.** Squirt cold 5% TCA around the inside of the filter 3x slowly – this should give you a volume of ~2mL TCA. Let stand for 2 minutes, then turn on the pump and filter it through.
8. With the pump still on, **rinse the towers 3x with TCA.**
9. **Remove the towers** and **rinse the exposed filters 3x with TCA.**
10. Turn off the pump, remove the filters, and **place filters into scintillation vials**.
11. Quickly **rinse the towers and the tops of the Hoeffer boxes** 3x with DI water.
12. **Set up the Hoeffer box and towers**, as in Step 5.
13. **Filter the Thymidine samples through** HA filters at <0.3atm**,** as in Step 6.
14. **Add TCA to kill the incubation,** as in Step 7.
15. **Rinse the towers 3x with TCA,** as in Step 8.

16. **Rinse the filters 3x with TCA,** as in Step 9.

17. **Place the filters into scintillation vials.** You're almost done for the night, just clean up!

III. Clean-up Protocol: Complete immediately after Part II on SPOT Night

1. Carefully pour the radioactive filtrate out of the Hoeffer boxes and into the liquid waste container. Use the funnel!

2. Rinse the following 3x with DI water: Hoeffer boxes, the tops of the Hoeffer boxes, the filtration towers, the box used to separate out filters, the tweezers. Set the towers and Hoeffer boxes and tops inside the RADs hood to dry overnight.

3. Spray areas A-K liberally with NoCount, and wipe them down.
    A. Hood counter (also open up the hood and clean beneath it)
    B. Floor under the hood (this includes the surface of the acid storage cabinets under the hood; be especially thorough with the handles)
    C. Surface of the liquid waste container
    D. Floor below the liquid waste container
    E. Dry waste container
    F. Rad work counter top (usually covered with paper, so just spray around the sink)
    G. Floor below the rad work counter top and fridge (again, this includes the cabinet surface and handles)
    H. Inside the sink
    I. Floor below the sink
    J. RADs fridge door
    K. RADs incubator door
    L. Lab door handle leading to the Heidelberg lab
    M. BLANK

4. Complete a wipe test for these surfaces.
    a. Tear up little pieces of paper towel and soak them in DI water.
    b. Using the tweezers, wipe down each surface with one towel piece, and place the it in the corresponding scintillation vial.
    c. You should discard the towel pieces, change DI water, and rinse the tweezers every 3-4 letters.
    d. Note that L and M are blanks! Do not use NoCount on the door to the Heidelberg lab, and for M, just put a wet piece of paper towel directly into a scintillation vial.

5. Add 5mL scintillation fluid to each scintillation vial using the fixed-volume dispenser on the RADs bench. Tightly cap the vials and shake them HARD.

6. Store the scintillation vials in the dark for at least 3 hours, preferably overnight, using the cabinet under the RADs bench.