**Ideally the AFS schedule starts on a Friday**

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| --- | --- | --- | --- | --- | --- |
|  | **Mon** | **Tues** | **Wed** | **Thur** | **Fri** |
| **Week 1** | **Schedule perfusion by this time** |  | **Day of Perfusion** | **Cryoprotect Tissue** | **Day 1: Freezing and UA infiltration** |
| **Week 2** | **Day 2: Make Lowicryl and Infilitrate** | **Day 3: Embedding and UV polymerization** |  |  | **Day 4: Clean up and store tissue** |

#### Friday, Day 1: UA infiltration over the weekend

**Preparation**:

* 1.5% uranyl acetate (plenty for four chambers): UA dissolve very slowly, so you may want to prepare it few hours in advance or the day before and store in 4C.
* Filter 1.5% uranyl acetate using 0.2 µm 25 mm syringe filter and 60mL syringe

**PROCEEDURE**

1. Draw diagram in notebook to organize tissue locations for run.
2. Notch one plastic basket in each can or otherwise distinguish a starting cylinder.
3. Place metal cans with four plastic baskets each filled with UA in acetone into AFS just before cool down.
4. Place one extra can for inserting and sorting tissue.
5. Select Lowicryl HM20 program. Start it, then pause until tissue is added to AFS.

This program will leave specimens at –90°C for 30 hours, then raise temperature in 4°C increments per hour to –45°C.

1. One by one, take each cryotube in cryogloved hands from liquid nitrogen container and very quickly uncap and transfer tissue into empty sorting can in AFS. Minimize time outside of LN2 or AFS chamber. Once in can use cryotube label to locate appropriate basket and sort tissue. Tally piece of tissue in notebook. Repeat for each piece of tissue.
2. Place acetone in metal cans into AFS.
3. Fill AFS to full with LN2 for the weekend.

You must wait 2-3 days (until Monday) before going on to Day 2 of AFS.

**Monday, Day 2: Make Lowicryl and Infiltrate**

1. Use pre-cooled acetone to wash samples with anhydrous acetone, 3 times, 15 minutes per wash (if it is not precooled, chill for 30 minutes prior to washing). Use glass pipettes to transfer solutions. One pipet for removing fluid, and one for adding acetone to dishes. If condensation gathers, switch to a new pipet. Try not to accumulate ice in acetone dish.
2. Prepare resin as follows during the 1st 15 min wash or during initial cooling. Wear gloves. Initiator C is a neurotoxin. Be extremely careful, and use black neoprene gloves while handling it.

Use the **Lowicry HM20 Embedding Kit**

(Electron Microscopy Sciences- Cat#14340)

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| --- | --- | --- | --- | --- |
| # of Cylinders: | 1 | 2 | 3 | 4 |
| Crosslinker D | 4.47 g | 7.45 g | 10.43 g | 13.41 g |
| Monomer E | 25.53 g | 42.55 g | 59.57 g | 76.59 g |
| Initiator C | 0.15 g | 0.25 g | 0.35 g | 0.45 g |

**Be extremely careful! Initiator C is a neurotoxin!**

1. Label new empty 250ml Lowicryl bottle with date.
2. Attach a glass pipet to the liquid nitrogen tank’s tubing. Close stopcock.

c) Tare scale with empty 20ml scintillation vial (other bottles will reach the maximum weight on that scale).

d) Measure Crosslinker D and add to labeled Lowicryl bottle.

e) Re-tare scintillation vial and measure out Monomer E.

e) Once the first two components are added, turn on nitrogen, slightly open stopcock, and gently bubble nitrogen gas into Lowicryl bottle for 10 minutes. (It should be about time for the next acetone wash.

f) Cut a plastic scoop in half. Tare a small plastic weigh boat inside of a larger weigh boat for second containment. Put on neoprene gloves. Carefully open and weigh out Initiator C using plastic scoop.

g) When Lowicryl is done bubbling, remove pipet and carefully add Initiator C. Return pipet and bubble for another 10 minutes. (gentle stirring helps to mix it faster, but the nitrogen helps to get oxygen out of the liquid)

h) Make 1:1 lowicryl:acetone and 2:1 lowicryl:acetone dilutions. You will need 4ml for each can. Label them clearly and add them to the AFS to chill for at least 30 minutes before washes.

i) Clean the scale well with methanol or acetone after use!

The AFS will pause at -45C and wait for the UV light step, so running the program as is is fine. Fill the AFS with more liquid nitrogen if it is getting low. I find that it takes about 5 dewars full to get through the second week.

1. To do infiltration with Lowicryl HM20 resin at -45oC, change buffers every two hours as follows. Be sure to pre-cool the buffers for at least thirty minutes each time before changing. The waiting periods are a good time to make labels. Add tissue to database. Make sure you make the embedding date for tomorrow.
   1. *Lowicryl/acetone 1:1* 2 hours
2. *Lowicryl/acetone 2:1* 2 hours
3. *Pure Lowicryl* 2 hours
4. *Pure Lowicryl* Over Night

If there’s room, add lowicryl to AFS chamber for tomorrow’s embedding.

#### Tuesday, Day 3: Embedding and UV-Polymerization

1. Make sure chilled lowicryl is available. If adding today, bubble with nitrogen first. Place enough beem capsules into the chamber to do the first wave of embedding. Chill them down for 20 minutes. Have labels ready. Have available nub-nosed forceps, small orange or green foreceps, a paintbrush, and pipets. Chill all tools before touching tissue.
2. Add a small amount of lowicryl to capsules, avoiding condensation. Carefully match labels with tissue, moved and center each piece in capsule with brush.
3. Insert rolled label with sharp forceps, top off capsule with lowicryl, and close cap with nub-nosed forceps.
4. Once completed, put UV light in place, plug in, and screw in screws gently with screwdriver.
5. Check that program has restarted on its own. \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

#### Friday, Day 4: Clean Up

Take out the blocks and clean everything.

1. Set AFS Machine to burn out program (60C) for 3 days to get rid of excess liquid nitrogen. Pour a small amount of acetone into chamber to clean off lowicryl. Wipe out lowicryl.