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PROCEDURE FOR THE PERMEABILIZATION AND CRYOBIOLOGICAL PRESERVATION OF DROSOPHILA EMBRYOS

K. W. Cole
P. D. Schreuders
A. P. Mahowald
Peter Mazur

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8.	Glycine	Sigma	#G-6388
9.	n-Heptane, HPLC grade	Fisher Chemicals	#H350-1
10.	Instant Drosophila Medium Formula 4-24	Carolina Biological Supply Company	#17-3204
11.	Magnesium chloride	J. T. Baker	#2444
12.	Magnesium sulfate	J. T. Baker	#2500
13.	DL-Malic acid	Sigma	#M-0875
14.	Polyvinylpyrrolidone (PVP)	GAF Chemical Corp.	Plasdone C-30
15.	Potassium hydroxide	J. T. Baker	#3146
16.	2-Propanol, reagent grade	Fisher Chemicals	#A416-500
17.	Rhodamine-B	Sigma	#R-6626
18.	Sodium chloride	Fisher Chemicals	#S-271
19.	Sodium citrate	Fisher Chemicals	#S-278
20.	Sodium hydroxide	J. T. Baker	#3722
21.	Sodium phosphate, monobasic	Mallinckrodt	#7892
22.	Succinic acid	Sigma	#S-7501
22.	Sucrose, enzyme grade	Bethesda Research Laboratories	#5503UA
23.	Yeast, baking (active dry)	Fleischmann's Yeast, Inc.	

F. Solutions

Note: The formulations for the solutions below are found in Section VII.

1. Agar, 2%, in water
2. Agar, 3%, in D-20
3. 1-Butanol/n-Heptane (0.3% butanol in heptane)
4. Clorox, 50% in glass distilled water
5. D-20 *Drosophila* culture media
6. Loading solution (2.0 M ethylene glycol in D-20)
7. Rhodamine B (0.1% in D-20)
8. Saline (0.139 M NaCl [~250 mmol/Kg])
9. Vitrification solution (8.5 M ethylene glycol + 10% PVP in D-20)
10. Warming solution (0.75 M sucrose in D-20)
11. Yeast paste
12. Yeast suspension (stock)

III. PREPARATION FOR THE EXPERIMENT

A. Have Available at the Wild Stereo Microscope

1. Wash bottle containing glass distilled water.
2. 400 ml beaker containing distilled water for rinsing camel hair brush.
3. Modified camel's hair brush trimmed to <10 bristles (for transferring eggs from filter to filter).

4. Watchmaker's forceps (for handling PC filters).
5. Large Petri dishes containing Whatman filters.
6. PC (polycarbonate) Filters (plain and gridded).

B. Have Available at the Water Bath {5} (Section VIII-A)

1. Bronze block
2. Controlled temperature bath
3. Digital thermometers (2 ea) with thermocouple junction in water and in bronze block
4. Refrigerated recirculator

IV. Collection, Incubation, and Staging of Eggs

A. The Day Prior to An Experiment:

Since this protocol will be used in *Drosophila* laboratories, we give only selected details on the rearing of the flies. We maintained them by the method of Travaglini and Tartof {6}.

1. Adult flies are reared in 18 × 18 × 12 inch plexiglass boxes. A nylon screen covers the back cutout and a cloth sleeve is attached to the front cutout.
2. The maintenance food source consists of Formula 4-24 Instant Drosophila Media (Carolina Biological Supply) plus Fleischmann's Active Dry Yeast.

   The daily food source is prepared by placing 50 g of Formula 4-24 Instant Drosophila Media into a 2S size styrofoam tray (5.75" × 8.25"). After the medium is moistened with 200 ml of glass distilled water, 2.0 g of Fleischmann's Dry Yeast is sprinkled on the surface.

   Over a weekend, two 4D size styrofoam trays containing 170 g of Formula 4-24 moistened with 400 ml glass distilled water and sprinkled with 4 g of active dry yeast are added on Friday afternoon and discarded on Monday morning. For long weekends or holidays, a third 4D size tray is added to the box.
3. At 9:45 a.m. the day of collection, two 2S size styrofoam trays containing 2% agar smeared with 2 g of yeast paste are placed in the box where they remain for 60 min. This is an egg "clean up" procedure for the female flies. The eggs are not used because of the wide range of stages of development present.
4. At 10:45 a.m., to obtain eggs for experimental use, the two trays are replaced with two similarly prepared agar/yeast trays. They are kept in the box for 60 min.

5. At 11:45 a.m., the trays are removed from the box and the eggs and yeast are washed into a stack of three 220 mm diameter sieves with distilled water. The top sieve size is 425 microns and will remove large unwanted material such as flies that may have become stuck in the yeast. The middle sieve size is 300 microns and removes smaller unwanted material. The bottom sieve size is 44 micrometers and will contain the eggs but not the yeast.

6. The eggs on the bottom sieve are rinsed thoroughly with distilled water to remove any yeast, etc. Any larvae mixed in with the eggs can be removed later.

7. When the eggs are clean, the bottom sieve is turned on edge and the eggs are washed onto a dampened 9 cm Whatman #1 filter with the aid of a stream of water from a wash bottle. During the transfer of the eggs from the sieve, the filter is under house vacuum. This inhibits any overflow of eggs and water, keeping all the eggs on the filter.

8. The filter containing the eggs is then placed in a Falcon #1001 Petri dish. At this time, 8-25 mm agar discs are cut into a #1001 Petri dish containing 2% agar. These are carried to the Wild scope bench.

9. At about 11:55 a.m., the eggs are transferred by modified camel's hair brush to 25 mm PC gridded filters. (During the transfer, carried out under the scope, the filters are supported on the agar discs.) Several hundred eggs are spread on each of 4 to 8 PC filters to form monolayers.

10. These PC filters are transferred by forceps to a clean 9 cm Whatman filter which has been moistened with distilled water. The eggs are then held at 24° C until 2:30 p.m., at which time they are placed in the 17° C water bath where they remain until 8:30 a.m. the following morning {7}.

B. On Experiment Day:

1. At 8:30 a.m., the Petri dish containing the egg samples is transferred from the 17° C bath to 24.5° C and maintained at 24.5° C ±0.5° until the eggs reach the proper stage.

2. To follow development, one PC filter is removed and the embryos dechorionated, or dechorionated and permeabilized (see Section V-B). This sample is examined about every 15 or 30 min until about 50% of the embryos are at Stage 14 and 50% Stage 15 {10}, a time that can and should be determined to within ±15 min. That time is defined as "zero" time, and corresponds to a 12.5 hrs developmental stage {3}.

3. All samples are then held at 24.5° C for "0" + 0.75 to "0" + 1.25 hrs (the time and temperature are critical {11}). We now use "0" + 1.0 hr.

4. All samples are then placed in a 4° C refrigerator until permeabilization is to be initiated.

V. THE EXPERIMENTAL PROCEDURE

A. Apparatus Required

At the Wild Stereo Microscope Bench:

1. Several packages of #3001 small Petri dishes.
2. #3001 Petri dishes containing ~1 ml D-20 solution (15 ea).
3. #1001 Petri dishes containing Whatman filters.
4. Sterile pipettes (1.0 ml, 5.0 ml, and 10.0 ml).
5. Box of 5-3/4 in. and 9 in. sterile Pasteur pipets and rubber bulbs.
6. Flask of saline solution, 0.139 M.
7. Box of Kimwipes.
8. Box of utility wipes.
9. #3001 Petri dish containing 3.0 ml 0.1% Rhodamine B stain.
10. Modified camel's hair brush (for transferring eggs from filter to filter).
11. Watchmaker forceps (for handling PC filters).
12. Two to three tissue culture flasks of sterile D-20 solution (~260 OSM).
13. Millipore Swinnex PC filter holder (top, bottom and Teflon gasket).
14. Stop watch and two timers.
15. Plain and gridded polycarbonate (PC) filters.
16. Petri dish (#3001) containing a plain PC filter floating on 1.0 ml D-20. (Some experiments may also require a dish containing a second plain PC filter on D-20).
17. Wash bottle containing glass-distilled water.
18. Bibulous paper.
19. Distilled water for rinsing camel hair brush.
20. Flask of warming solution (0.75 M sucrose in D-20).
21. Flask of loading solution (2.0 M ethylene glycol in D-20).
22. Flask of vitrification solution (8.5 M EG in 10% PVP in D-20).
23. Large glass Petri dish and cover (140 × 20 mm)

At Vacuum apparatus (where permeabilization is carried out):

1. Vacuum apparatus with vacuum flask and tubing set up with Leur lock to accept Swinnex (Section VIII-B).
2. For each permeabilization run, four 50 ml graduates containing: (a) 30 ml of 50% (v/v) Clorox in water, (b) 20 ml of isopropanol (2-propanol), (c) 30 ml of 0.3% 1-butanol in n-heptane, and (d) 15 ml of pure n-heptane.
3. Three 30 ml syringes for: (1) Clorox solution, (2) water rinse and alcohol, and (3) the alkane solution and pure alkane. These syringes should be appropriately labeled to ensure that they are not interchanged. We color code (1) and (3) yellow and red respectively, and label syringe (2) ***H_2O/Alcohol***.

4. Four Swinnex tops color coded or labeled to match the syringes in (3), above. The first (color coded yellow) is used for the Clorox. The second is used for the water rinse. The third is used for the isopropanol. And the fourth (color coded red) is used for the butanol-heptane and the pure heptane. The latter two are fitted with a 50 μl capillary to serve as a vent {12}.
5. An extra, clean, Swinnex Teflon gasket.
6. Box of Kimwipes.
7. Covered 400 ml beaker and 1 liter graduate containing glass distilled water.
8. Timer reading to <1 second increments.
9. Extra 50 μm capillary micropipettes.
10. Supply of 9-inch Pasteur pipettes and rubber bulb.

On bench near vacuum apparatus:

1. Dissecting scope.
2. Beaker, with flowing distilled water, to flush Clorox syringe, Swinnex top, and gasket after a permeabilization run.

B. Steps to Dechorionate and Permeabilize *Drosophila* Eggs:

The permeabilization procedure involves dechorionation with hypochlorite, an extensive water rinse, exposure to isopropanol to provide a medium compatible with both water and alkane, air drying to evaporate the alcohol, exposure to a butanol-heptane mixture to effect the actual permeabilization, a "chase" with pure heptane to slow further permeabilization, and finally transfer to D-20 to return the embryos to an aqueous environment. All except the last step occur within the Swinnex filter holder by the successive addition of fluids to a 30 ml syringe above the holder. To avoid introducing an air gap, new liquid is added to the syringe just before the final exit of the previous liquid.

- **Setup**

1. Add 1.0 ml D-20 to each of ~15 #3001 Petri dishes.
2. Prepare 4 pipettes (5-3/4" Pasteur), topped with rubber bulbs, and containing ~1.0 ml D-20 in each.
3. Place one polycarbonate (PC) filter on top of D-20 in a Petri dish (from step #1). This filter will be used later for assessing rhodamine staining.
4. The PC filter containing the "0" + 0.75 to "0" + 1.25 hr eggs to be permeabilized (Section IV-B, above) is weighed and, if necessary, eggs are removed with a camel's hair brush to obtain a total egg weight of 5-8 mg (approx 500-800 eggs) {13}.

5. To start the permeabilization process, place the PC filter (usually gridded {14}) and its adherent embryos on the Swinnex bottom and make a sandwich by placing a plain PC filter on top of the eggs. The Teflon gasket is then positioned and the top dome of the Swinnex screwed in place. The Swinnex, forceps, and a Petri dish (#3001) containing 1.0 ml D-20 are then carried to the vacuum apparatus. A second Petri dish containing 1.0 ml D-20 is placed by the dissecting scope.

6. At the vacuum apparatus, check the flow meters in the house vacuum line to be sure the first meter, R-6-25-B, is reading 8, and the second meter R-2-25-B, is reading 18 {15}. Place the Swinnex containing the filter sandwich on the Leur lock tip of the apparatus and screw a 30 ml glass syringe onto the Swinnex.

- **Dechorionation**

1. Pour 30 ml of 50% Clorox solution into the yellow color-coded 30 ml glass syringe and turn the apparatus needle valve until the Clorox just starts to drip into the vacuum flask. Then adjust the flow rate with the needle valve so that it takes 2.5 min for the 30 ml of Clorox to run through the Swinnex.

2. At the end of the Clorox run, allow a syringe full of glass-distilled water to pass through the system. Then remove the 30 ml syringe, the Swinnex dome and gasket and place them in running distilled water in a beaker in the sink.

3. Attach a clean, dry Swinnex dome (marked H_2O) and its gasket to the bottom of the Swinnex (still attached to the vacuum apparatus) and attach a clean 30 ml syringe to the dome.

4. Add 25 ml glass-distilled water to the syringe and open the valve an additional half turn to allow the water to run rapidly through the system. Repeat this step five more times, at which point the filter sandwich has been washed with a total of 150 ml of water {16}.

5. At the end of the water rinse, leave the valve at the position in 4, above, to allow air flow through the filter sandwich, but remove the syringe, the Swinnex dome, and the gasket. Now, dry the syringe by shaking out any excess water and drying the inside with a paper towel.

- **Isopropanol Step and Air Drying**

1. Place a dry vented dome (marked "alc") with its gasket, on the Swinnex bottom (which is still on the vacuum apparatus), and place the dried syringe on the Swinnex.

2. Add 20 ml of isopropyl alcohol to the syringe and allow the alcohol to run through in ~30 sec {17}.

3. Start timer clock at zero just as the last of the alcohol leaves the syringe. Then allow air to pass through the system for 2 min. Keep the steel ball in the flow meter at 18 during the drying time {18}.

- **Permeabilization**

1. During the 2-min air drying, remove the syringe, Swinnex dome, and gasket from the Swinnex bottom and place them in a hood to evaporate the alcohol. Then attach another vented Swinnex dome (color coded red) and its gasket to the Swinnex bottom, and attach a 30 ml syringe (color coded red) to the Swinnex dome.
2. At the end of the 2-min drying time, add 30 ml of 0.3% butanol in heptane to the 30 ml syringe. Adjust the flow so that it takes 90 ±2 sec for the 30 ml of solution to pass through the Swinnex {19}.
3. Immediately follow the butanol-heptane solution with a "chase" of 15 ml of pure n-heptane {20}.
4. Then remove the syringe from the Swinnex, remove the entire Swinnex from the apparatus, and open the Swinnex, and remove the filter sandwich with forceps. Insert the sandwich into a #3001 Petri dish containing ~1 ml D-20 and place the dish under a dissecting scope. Gently and carefully separate the filters so as not to loosen the eggs. The top filter is placed in the second Petri dish containing D-20. The time for step 4 is ~15 sec and should be kept as brief as possible.

   IT MUST BE EMPHASIZED THAT FROM THIS POINT ON, THE NOW PERMEABLE EMBRYOS MUST BE KEPT IN CONTACT WITH AN ISOTONIC AQUEOUS SOLUTION.
5. After swirling the Petri dishes, aspirate off the D-20 solution from each dish with a 5-3/4" Pasteur pipette, and then immediately add more D-20 alongside each filter. (Do not disturb the eggs by placing the D-20 on them.) Repeat this procedure twice more.
6. Transfer the filters to other #3001 dishes containing D-20. Repeat this transfer twice more {21}.

   This completes the permeabilization procedure.

C. Recovery, Staining, and Splitting Samples

1. A higher percentage of the now permeabilized embryos survive if they are given a 45 min recovery period at room temperature prior to the initiation of the vitrification procedure {4, Table 3}. The 45 min incubation can be carried out on the bench top provided that the #3001 dishes containing the filters are capped to prevent evaporation. (We usually place the dishes in the high humidity box [see Section VIII].)

PROCEDURE FOR THE PERMEABILIZATION AND CRYOBIOLOGICAL PRESERVATION OF DROSOPHILA EMBRYOS*

K. W. Cole[1], P. D. Schreuders[2], A. P. Mahowald[3], and Peter Mazur[1‡,2]

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[1]Oak Ridge National Laboratory, Biology Division, P.O. Box 2009, Oak Ridge, Tennessee 37831-8077
[2]University of Tennessee–Oak Ridge Graduate School of Biomedical Science, Oak Ridge, Tennessee 37831-8077
[3]University of Chicago, Department of Molecular Genetics and Cell Biology, Chicago, Illinois 60637

I. INTRODUCTION

We describe below the detailed protocol developed in our laboratory at Oak Ridge for the permeabilization and cryobiological preservation of embryos of *Drosophila melanogaster*, Oregon R strain. The protocol is supplemented by notes containing two sorts of information. One category includes references to the appropriate portions of our published papers {1-4} giving the scientific rationale and experimental basis for important steps. The other category is concerned with the criticality of certain steps and the precision with which they need to be performed. As an aid to investigators, we list even ordinary pieces of equipment. Brand names and model numbers are given where it is either important or convenient for readers to know precisely what we use.

II. THE APPARATUS AND REAGENTS

A. Equipment

1.	Stereo microscope	Wild	#TYP 355110
2.	Stereo microscope (Near permeabilization apparatus)		
3.	Water bath, 18° C	Lab-Line Instruments	#13100
4.	Digital thermometer	Fluke	#2176A
5.	Digital thermometer	Fluke	#2190A
6.	Stirring hot plate		

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*A preliminary version of this protocol was distributed at a workshop on *Drosophila* cryobiology at the *Drosophila* Research Conference, San Diego, April 3, 1993. Supported by the Eukaryotic Genetics Program, National Science Foundation (Grant DMB8520453) and by the Office of Health and Environmental Research, U.S. Department of Energy under contract DE-AC-05-840R21400 with Martin Marietta Energy Systems, Inc.

‡To whom inquiries should be addressed.

G. Warming and Removal of Vitrification Solution

1. At the conclusion of the storage period, the eggs are warmed at a high rate by transferring the sample PC filter as abruptly as possible {24} from the liquid nitrogen to about 40 ml of a solution of 0.75 M sucrose in D-20 at 24° C. Stirring is stopped just before transfer to avoid dislodging the eggs).
2. After 10-60 sec, the filter is transferred to a #3001 dish containing 1-2 ml of the same solution and held in that solution so that the total time in the sucrose is 2 min {25}.
3. After 2.0 min, remove the filter from the sucrose, wipe the undersurface on a Kimwipe. Then place it in D-20 and carefully add several drops of D-20 to the eggs. Transfer the filter to a second dish and then a third dish of D-20, to remove the ethylene glycol and sucrose from the eggs and the surrounding medium.

H. Incubation and Determination of the Percentage Hatching

1. Place the PC filter face up on the surface of a 1 ml layer of 3% agar in a #3001 dish. (The agar is made up in D-20 {26}).
2. Add approx 10 μl of a 50-fold dilution of the yeast stock suspension (Section VII) around the margin of the PC filter.
3. This last dish is capped and placed on filter paper moistened with 0.139 M NaCl in a 100 mm Falcon #1001 Petri dish. The larger dish in turn is capped and placed in a chamber maintained at 24-25° C near 100% relative humidity (Section VIII) {27}. Percent hatching is determined 15 to 20 hrs later.

I. Development to Adulthood

1. Transfer portions of the agar that hold the larvae along with the PC filter to a 15 x 95 mm shell vial (Kimble 60930) containing about 7 ml of the desired *Drosophila* medium (we use a 1:3 [w/w] mixture of Carolina Biological Supply medium and water), and incubate the vials at 24-25° C and 60-65% relative humidity. We limit the number of larvae in each vial to about 50.
2. After 2 days incubation add 10 μl of a 10-fold dilution of the stock yeast suspension to each vial.
3. Continue incubation for an additional 10-11 days by which time nearly all development to adulthood is completed. (The development of larvae from cryopreserved samples appears to lag that of untreated controls by about 1 day.)

VI. NOTES AND REFERENCES

{1} Mazur, P., Cole, K. W., and Mahowald, A. P. Critical factors affecting the permeabilization of *Drosophila* embryos by alkanes. *Cryobiology* **29**, 210-239 (1992).

{2} Mazur, P., Schneider, U., and Mahowald, A. P. Characteristics and kinetics of subzero chilling injury in *Drosophila* embryos (with an Appendix by T. J. Mitchell). *Cryobiology* **29**, 39-68 (1992).

{3} Mazur, P., Cole K. W., Hall, J. W., Schreuders, P. D., and Mahowald, A. P. Cryobiological preservation of *Drosophila* embryos. *Science* **258**, 1932-1935 (December 18, 1992).

{4} Mazur, P., Cole, K. W., Schreuders, P. D., and Mahowald, A. P. Contributions of cooling and warming rate and developmental stage to the survival of *Drosophila* embryos cooled to -205° C. *Cryobiology* **30**, 45-73 (1993).

{5} The brass block, the top of which is just clear of the bath water, provides good thermal contact between the water and the bottom of the Petri dish. When we have a second Petri dish of samples, we place them on stainless steel test tube racks in the water bath.

{6} Travaglini, E. C., and Tartof, D. *Drosophila Information Service* **48**, 157 (1972).

{7} The 10:45 a.m.-11:45 a.m. egg collection and the maintenance at 17° C from 2:30 p.m. to 8:30 a.m. the next day is simply a convenient time-temperature regimen to obtain approx 11 hr eggs early on the day of the experiment. The development rate of embryos at 17° C is about half that at 24° C {8}. The 17° C exposure appears to be without adverse effect since Steponkus' group {9} has obtained similar cryopreservation results with embryos allowed to develop continuously at 25° C.

{8} Ashburner, M., and Thompson, J. N. Jr. The laboratory culture of *Drosophila*. In "The Genetics and Biology of *Drosophila*" (M. Ashburner and T. R. F. Wright, Eds.), Vol. 2A, pp. 1-109. Academic Press, London, 1978.

{9} Steponkus, P. L., Myers, S. P., Lynch, D. V., Gardner, L., Bronshteyn, V., Leibo, S. P., Rall, W. F., Pitt, R. E., Lin, T.-T., and MacIntyre, R. J. Cryopreservation of *Drosophila melanogaster* embryos. *Nature* **345**, 170-172 (1990).

Steponkus, P. L., and Caldwell, S. Cryopreservation of *Drosophila melanogaster* embryos by vitrification. *Cryobiology* **29**, 763-764 (1992) (Abstract).

{10} Wieschaus, E., and Nüsslein-Volhard, C. Looking at embryos. In "*Drosophila*–A Practical Approach" (D. B. Roberts, Ed.), pp. 199-227, IRL Press, Oxford, 1986.

{11} We {3} and Steponkus and Caldwell {9} have determined that survival after vitrification procedures is critically dependent on the developmental stage of the embryos at the initiation of the ethylene glycol exposure. The developmental rate is too sensitive to small variations in temperature to adequately control developmental stage by timing from egg laying; hence, our procedure is to accurately define a "zero"

time as the time 50% of eggs are Stage 14 and 50% Stage 15 {10}. At the onset of permeabilization, the embryos are "0"+ 0.75 to "0" + 1.25 hrs. Permeabilization and the subsequent recovery treatment take about 1.25 hrs, so that at the time the embryos are subjected to 2 M ethylene glycol, they are "0"+ 2 to "0"+ 2.5 hrs of age. These are the times shown in Fig. 1 of {3} to yield peak hatching and adult survivals.

{12} The capillary vents were included early in the development of the procedure to eliminate trapped air in the Swinnex top that might prevent solutions from contacting the eggs.

{13} There is evidence {1, Table 4} that the percentage of eggs permeabilized decreases when the number of eggs on a PC filter exceeds 900.

{14} Gridded filters facilitate the subsequent determination of percent hatching.

{15} We use the flow meters to ensure that a given position of the needle valve produces about the same flow rate of solutions through the vacuum assembly. However, they are probably not necessary if the vacuum source is reasonably constant.

{16} Experiments reported in {1, p. 217} showed that this quantity of wash water was required to reduce the concentration of residual Clorox in the filter sandwich by a factor of 10,000. We do not know whether less effective washing and higher residual hypochlorite would be deleterious to subsequently permeabilized embryos.

{17} The purpose of the isopropanol is to remove all vestiges of water prior to subsequent introduction of alkane.

{18} ***The 2 min air-drying is a critical step.*** Its purpose is to evaporate nearly all isopropanol and, thereby, prevent a significant and variable alteration in the alcohol-alkane ratio in the next step (see {1, pp. 219-220}). The flow meter reading of 18 produces a flow rate of ~1.25 liters of air/min. Flow rates of 0.75 to 1.75 ℓ/min did not alter the percentage permeabilized {1, p. 226}.

{19} ***This is a critical step at which actual permeabilization occurs.*** The effectiveness of permeabilization increases with the concentration of alcohol in the alkane and the length of exposure. But, so also does injury. Both also depend on the nature of the alcohol and the alkane. In our hands, the cited conditions maximize permeabilization while minimizing injury {1, Fig. 11 A and Table 5}. The data in Ref. {1} were obtained on 12 hr embryos. The exposure to 0.30% butanol-heptane for 90 sec yields appreciably higher survivals for the 15-hr embryos now being used {3}. It is important that the age of the embryos not exceed that recommended since the efficiency of permeabilization drops off abruptly in older embryos {3, Fig. 1}.

{20} The passage of pure alkane produces an abrupt decrease in the concentration of butanol to which the embryos are exposed. The effect of this is to prevent excessive permeabilization and loss in viability during subsequent processing {1, p. 237}.

{21} During steps 5-6 the residual alkane evaporates {1, Fig. 3}.

WE EMPHASIZE THAT THE 45 MIN RECOVERY PERIOD IS INCLUDED IN THE EMBRYOS' DEVELOPMENT TIME SINCE "ZERO" TIME (see Section IV-B above and {3, Fig. 1 and Table 1}.

2. During the 45-min recovery time, a subsample of the eggs from each permeabilization run is subjected to rhodamine staining to assess the effectiveness of permeabilization. If desired, other subsamples can also be created in several ways.

   (a) If these subsamples are to be subjected to vitrification, it is important that the eggs not be loosened from the PC filter surface, else they are likely to become dislodged during vitrification or subsequent warming. To meet this requirement one can either cut the filter in half or thirds, or use eggs that have adhered to the top half of the filter sandwich.

   (b) If treatments do not involve vitrification, subsamples can be created by transferring eggs by camel's hair brush to another PC filter.

3. One of the latter subsamples is used to assess the degree of permeabilization.

   (a) About 100 embryos are transferred to a PC filter which is floated face up for 5 min on a solution of 0.1% rhodamine B in D-20. During the 5-min staining, additional rhodamine solution is gently dropped on the surface of the eggs.

   (b) To remove excess stain, the filter is then transferred to three successive #3001 dishes with D-20 with wiping on Kimwipe between transfers.

   (c) The embryos are then categorized at 10-20 X under a stereo microscope into one of four groups: Ruby red, dark pink, light pink, and unstained. Normally, some 80% of the 15 hr embryos stain ruby red or dark pink {22}. If the percentage ruby red + dark pink is below 50%, either the permeabilization run was faulty (perhaps an air bubble in the Swinnex dome) or the embryos are too advanced. The optimum developmental stage for cryopreservation (14.5 - 15.0 hrs) lies close to the age at which the effectiveness of the permeabilization procedure drops precipitously {4, Fig. 8; 3, Fig. 1}.

D. Exposure to the Vitrification Solution

The exposure to the vitrification solution is carried out in two steps. First the embryos are allowed to equilibrate with the 2.0 M ethylene glycol "loading solution" for 30 min at room temperature. Second, they are exposed for five min near 0° C to a "vitrification" solution of 8.5 M ethylene glycol plus 10% polyvinylpyrrolidone (PVP). The purpose of the second step is to raise the intraembryonic concentration of ethylene glycol to near that of the surrounding medium by osmotic withdrawal of intraembryonic water.

1. At the conclusion of the 45-min incubation (step C-1), transfer the filter, or filters, face up with forceps, to a #3001 Petri dish containing 2.0 M ethylene glycol in D-20 at R.T. for 30 min. To reduce carry-over of D-20, the filter should be wiped on a Kimwipe before being placed in the 2.0 M ethylene glycol. Carefully add several drops of ethylene glycol to cover the eggs. Two or more filters, from the same run, can be processed in parallel in separate dishes of solution.
2. Towards the end of the 30-min exposure to 2 M ethylene glycol, 1 ml of 8.5 M ethylene glycol plus 10% PVP in D-20 that has been prechilled to 0° C in ice is transferred with a chilled pipette to a #3001 Petri dish resting in a 140 mm glass Petri dish containing ice and a small amount of water and in turn resting on ice.
3. The PC filter and its adherent eggs are removed from the #3001 dish containing the loading solution, wiped on the rim and then on a Kimwipe, and floated on the surface of the cold vitrification solution. About 0.1 to 0.2 ml portions of that cold glycol are then dropped gently on top of the eggs several times, taking care not to dislodge the eggs. The temperature of the ethylene glycol remains at ~5° C during this procedure.

E. Vitrification

1. Carry the ice-water dish with the filters to the site of the freezing apparatus. A few seconds before the conclusion of the 5 min exposure time, remove the PC filter from the vitrification solution, and place it face up on a piece of bibulous paper in a petri dish sitting on ice {23}. Quickly drag the filter across the paper in order to remove the excess solution.
2. Then abruptly plunge it into nitrogen slush or boiling liquid nitrogen using fine watchmakers forceps as the handle. The procedure for preparing nitrogen slush is given in Section VIII. Nitrogen slush was used in our publications to date {3 and 4}, but our more recent data suggest that survival using boiling liquid nitrogen is at least half as high, and the latter is considerably easier to use.

F. Storage

1. The vitrified samples should be stored in liquid nitrogen {24} in a suitable container. With one exception, in our experiments to date, the storage time at -196°C has only been some 30-60 sec. That one exception involved storage for 13 days with no loss in survival. Theory and limited experiments with other cell types provide assurance that storage for 30 or 300 years will produce the same results.

7.	Refrigerated recirculator	Neslab	Coolflow CFT-25
8.	Osmometer, vapor pressure	Wescor	#5500
9.	pH meter		
10.	Analytical balance		
11.	Top pan balance		

B. Modified Apparatus

Note: The parts lists for custom apparatus are found in Section VIII, along with the drawings and instructions.

1. Controlled temperature bath
2. Permeabilization apparatus
3. Vented Swinnex filter holder
4. Nitrogen slush apparatus
5. Humidity chamber

C. Glassware and Plasticware

1.	Large polystyrene Petri dishes, 100 × 15 mm	Falcon	#1001
2.	Small Petri dishes, wettable, 35 × 10 mm	Falcon	#3001
3.	Graduated cylinder (4 ea), 50 ml		
4.	Wash bottle of distilled water		
5.	Sterile pipets, plastic, 1 ml, 5 ml, 10 ml		
6.	Pasteur pipets, 5 and 9 in.		
7.	Pipets, glass, micro-capillary, 50 μm	Corning	#7099-S
8.	Syringe (3 ea), glass, Leur Lock, 30 ml		
9.	Flask, tissue culture, sterile, 50 ml	Falcon	#3013
10.	Styrofoam tray, size 2S, 5.75 × 8.25 inches		
11.	Styrofoam tray, size 4D		

D. Miscellaneous

1.	Bulbs (6x), rubber		
2.	Bibulous paper		
3.	Kimwipes, 4 1/2 x 8 1/2 in.		
4.	Filters, polycarbonate, 12 μm pore size, 25 mm	Poretics or Nuclepore/Costar	#11081 #110616

5.	Filters, polycarbonate (PC), gridded, 10 μm pore size, 25 mm	Nuclepore/Costar	#800056
6.	Filter, paper, qualitative, #1, 9 cm	Whatman	#1001 090
7.	Filter unit, tissue culture, 0.2 μm, and receivers, 150 ml	Nalgene	#150-0020 #455-0150
8.	Filter unit, tissue culture, 0.2 μm, and receivers, 500 ml	Nalgene	#151-4020 #455-0500
9.	Swinnex filter holder, 25 mm, (with Teflon gasket)	Millipore	SX00-025-00
10.	Swinnex filter holder, top only (4 ea)	Millipore	SX00-025-00
11.	Forceps, watchmaker	Dumont	#5
12.	Camel hair brush (trimmed)		
13.	Stop watch		
14.	Timers (2 ea)		
15.	Spatula		
16.	Sieve, USA standard, #40 (opening size=425 μm), 220 mm diameter		
17.	Sieve, USA standard, #50 (opening size=300 μm), 220 mm diameter		
18.	Sieve, USA standard, #325 (opening size=44 μm), 220 mm diameter		
19.	2% agar disks (8 ea), 25 x 3 mm		
20.	Approx 10 ea 3001 Petri dishes containing 1 ml of 3% agar in D-20 medium		
21.	2 ea 2S styrofoam laying trays (5.75" x 8.25") filled with 2% agar in water and smeared with a band of yeast paste		
22.	Face shield		

E. Chemicals

1.	Bacto-Agar	Difco	#0140-01
2.	1-Butanol, certified ACS	Fisher Chemicals	#A399-1
3.	Calcium chloride	J. T. Baker	#1332
4.	Household bleach	Clorox, regular	
5.	Ethylene glycol	Sigma	#E-9129
6.	D-(+)-Glucose	Sigma	#G-8720
7.	L-Glutamic acid	Sigma	#G-1251

{22} Another indication that embryos have been effectively permeabilized is that they will undergo extensive shrinkage in a solution of 0.75 M sucrose in D-20 in about 5 min at room temperature {1, Table 6}. We do not use the stained sample further as the Rhodamine appears slightly toxic.

{23} The mass of the ethylene glycol/PVP vitrification solution is kept at a minimum so as to maximize the rate of warming (see {24}). We have not tested whether blotting is required for 15 hr embryos, but we feel it prudent to include it.

{24} We have shown {4} that 12-hr embryos are killed by a few second's exposure to temperatures above -80° C, and that all are killed if warmed from -200° C to room temperature at 2000° C/min. Although we have preliminary indications that the 15-hr stage now being used may not be so sensitive to short exposures above -80° C and to warming rates of less than 100,000° C/min, prudence dictates that great care be taken that the filter samples be constantly maintained at liquid nitrogen temperatures until initiating warming, that the warming be effected by the method described, and that minimal time elapse between withdrawing the filter samples from LN2 and plunging them into the sucrose/D-20 warming bath. With respect to the last precaution, note that a filter held in room-temperature air will warm from -200° C to -80° C in about 3 sec.

{25} The purpose of the 2 min exposure to the 0.75 M sucrose in D-20 is to allow time for the efflux of the intraembryonic ethylene glycol without producing damaging osmotic swelling of the embryo {4, Table 3}.

{26} The agar is made with D-20 to provide an isotonic medium for the embryos. It may well be that 260 mosm NaCl or Drosophila Ringer's would serve as well. We use 3% agar because there is some evidence that newly hatched larvae crawl better on harder agar, but 2% agar in D-20 or in isotonic salt, would probably be equally satisfactory.

{27} To ensure that the permeabilized embryos have been maintained at near 100% relative humidity during incubation, we use an osmometer to measure the osmolality of 0.4 ml of D-20 in a capped test #3001 dish at the completion of incubation.

{28} Zalokar, M., and Santamaria, P. In "Syllabus of Experimental Methods, in *Drosophila* Embryology," EMBO Workshop, Gif-sur-Yvette (1977).

VII. SOLUTION FORMULATIONS

AGAR, 2% - IN WATER

Agar	20.0 g
Double Distilled Water	1000. ml

Preparation

Place water into a 2000 ml beaker. While agitating with a stirring bar, heat on hot plate until boiling. Add agar and stir until the mixture thickens and all lumps have disappeared. Pour through a kitchen strainer to remove any remaining lumps.

Pour into #1001 Petri dishes to a depth of ~3 mm. After allowing the agar to cool, place covered Petri dishes into plastic bags and store in refrigerator. Agar disks will be punched from the agar in these plates with a cork borer or comparable tool. Alternately, pour into 2S styrofoam trays for use in egg collection.

AGAR, 3% IN D-20

Agar	6.0 g
D-20 (double strength stock)	100.0 ml
Double Distilled Water	100.0 ml

Preparation

Mix D-20 stock solution with water. While agitating with a stirring bar, heat the solution on a hot plate until boiling. Add agar and agitate until the mixture thickens and all lumps have disappeared. Place 11 ml aliquots into sterile test tubes. Store in refrigerator. For use, melt the agar by placing a test tube in boiling water. Pipette ~1 ml agar into each of 10 #3001 Petri dishes.

1-BUTANOL/N-HEPTANE
(0.3% 1-Butanol in n-Heptane)

n-Heptane	199.4 ml
1-Butanol	0.60 ml

Preparation

Add n-Heptane to the mark of a 200 ml volumetric flask. Remove 0.60 ml with a 1.0 ml glass pipet. Add 0.60 ml of 1-Butanol using a 1.0 ml glass pipet.

CLOROX (50%)

Clorox Bleach	250.0 ml
Double Distilled Water	qs to 500.0 ml

Store in an amber bottle. This solution should be retained for no more than one week.

DROSOPHILA CULTURE MEDIUM (DOUBLE STRENGTH)
Modified D-20 (After Zalokar and Santamaria {28})

Solution A

L-Glutamic Acid	22.0 g
Glycine	11.0 g
DL-Malic Acid	1.0 g
Succinic Acid	1.0 g
Sodium Citrate ($\cdot 2\ H_2O$)	0.06 g
Glucose	4.0 g
$MgCl_2 \cdot 6\ H_2O$	4.0 g
$MgSO_4 \cdot 7\ H_2O$	5.0 g
$NaH_2PO_4 \cdot H_2O$	0.884 g
Double Distilled Water	800.0 ml

Solution B

$CaCl_2 \cdot 2\ H_2O$	1.8 g
Double Distilled Water	100.0 ml

Solution C

NaOH	15.0 g
KOH	15.0 g
H_2O	qs to 250.0 ml

Preparation

Prepare Solutions A, B, and C. Mix Solutions A and B together. Adjust this mixture to pH 6.8 using Solution C. Bring the volume to a total of 1100 ml using double distilled water. Filter sterilize and refrigerate this stock solution.

Dilute the stock solution 1:1 with double distilled water just prior to use. Determine and record the solution's osmolality. It should be 260 mosm/kg. Filter sterilize the solution into a 500 ml sterile 0.2 μm Nalgene filter unit and into 50 ml sterile tissue culture flasks. Refrigerate the solutions.

VITRIFICATION SOLUTION
18.68 Molal (8.5 M) Ethylene Glycol + 10% PVP in D-20

Ethylene Glycol	115.95 g
PVP (Plasdone)	21.95 g
D-20	100.00 g

Preparation

Mix components. Note that the solution is made up on a molal basis by adding 100 g of D-20. Filter sterilize (0.2 μm filter) and store refrigerated.

WARMING SOLUTION
0.75 Molar Sucrose in D-20

Sucrose	51.345 g
D-20	qs to 200.0 ml

Preparation

Mix components. Filter sterilize (0.2 μm filter) and store refrigerated.

LOADING SOLUTION
2.0 Molar Ethylene Glycol in D-20

Ethylene Glycol	24.828 g
D-20	qs to 200. ml

Preparation

Mix components. Filter sterilize (0.2 μm filter) and refrigerate.

RHODAMINE B STAIN
(0.1% Rhodamine B in D-20 {28})

Rhodamine B	0.1 g
D-20	qs to 100.0 ml

Preparation

Mix components. Filter sterilize (0.2 μm filter) and store refrigerated.

SALINE SOLUTION
(0.139 M Sodium Chloride in Water)

NaCl	8.142 g
Double Distilled Water	qs to 1000. ml

YEAST PASTE

Dry Baker's Yeast	60.0 g
Double Distilled Water	120.0 ml

Preparation

Warm the water to 45°C. Add water to the yeast, mixing with a spatula. The resulting paste should be the consistency of smooth peanut butter. If not, add additional water or yeast. Store refrigerated for up to one week.

YEAST SUSPENSION (STOCK)

Dry Baker's Yeast	0.31 g
Double Distilled Water	qs to 10.0 ml

Preparation

Warm the water to 45°C. Suspend yeast in the water. Store refrigerated for up to one week.

VIII. Custom Parts and Apparatus

A. Controlled Temperature Bath for Embryo Development after Laying

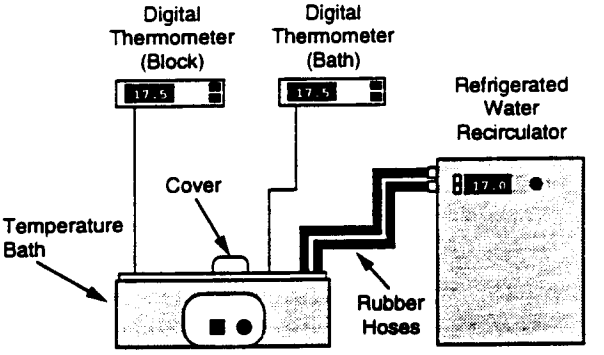
Parts and Equipment List

1. Digital thermometer	Fluke	#2176 A
2. Digital thermometer	Fluke	#2190 A
3. Refrigerated recirculator	Neslab	Coolflow CFT-25
4. Water bath	Lab-Line Instruments	#13100
5. Bronze block		

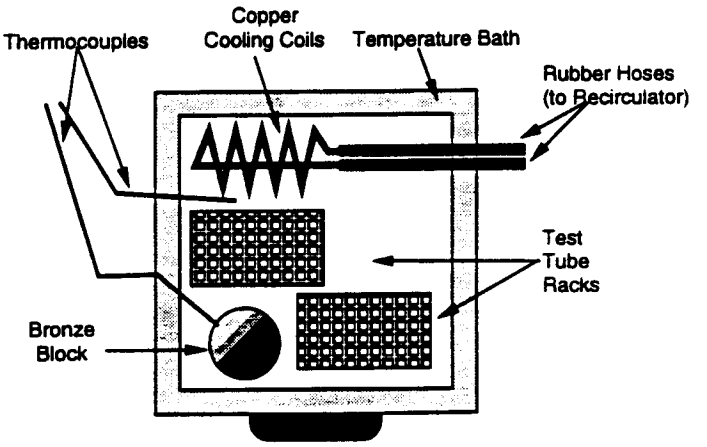
Instructions

The Neslab water chiller is set at 17° C. The temperature of the brass block in the water bath is 17.5 ±0.5° C.

Schematic



Assembled Apparatus



Temperature Bath (Top View)

B. Permeabilization Apparatus

Parts and Equipment List

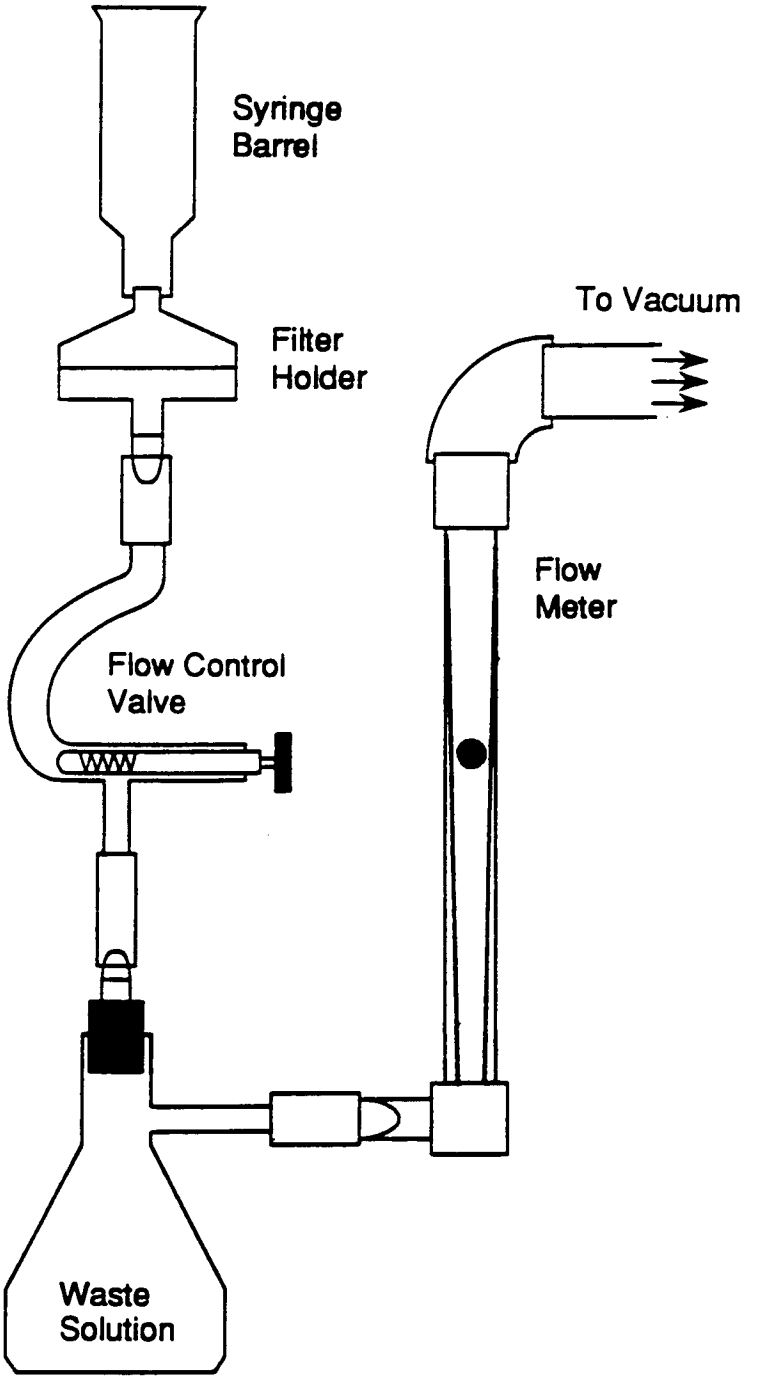
1. Syringe barrel (3 ea), glass, Luer lock, 30 ml		
2. Luer lock to hose adapter (2 ea)		
3. Ring stand (2 ea)		
4. Assorted clamps for ring stand		
5. Swinnex filter holder, 25 mm with Teflon gasket	Millipore	
6. Flow control valve	Fischer & Porter	
7. Needle, 17 gauge		
8. Flask, vacuum, 500 ml		
9. Stopper, #7, rubber, with diaphragm for needles, (for vacuum flask)		
10. Plastic disconnect		
11. Flow meter with stainless steel ball	Brooks, Inc.	#R-2-25-A
12. Valve, vacuum control	Fischer & Porter	
13. Flow meter with stainless steel ball	Brooks, Inc.	#R-6-25-B
14. Hose, vacuum		
15. Hose clamps, assorted		

Instructions

This apparatus is supported by a pair of ring stands. The first is used to support the apparatus from the syringe barrel to the R-2-25-A flow meter. The second stand is used to support the vacuum control valve and the remaining flow meter. To read correctly, the flow meters must be mounted vertically.

A plastic disconnect between the vacuum flask and the R-2-25-A flow meter allows removal of the vacuum flask when it becomes full, without disturbing the rest of the apparatus.

Schematic on Following Page


Syringe
Barrel
To Vacuum
Filter
Holder
Flow
Meter
Flow Control
Valve
Waste
Solution

C. Vented Swinnex-25 Top

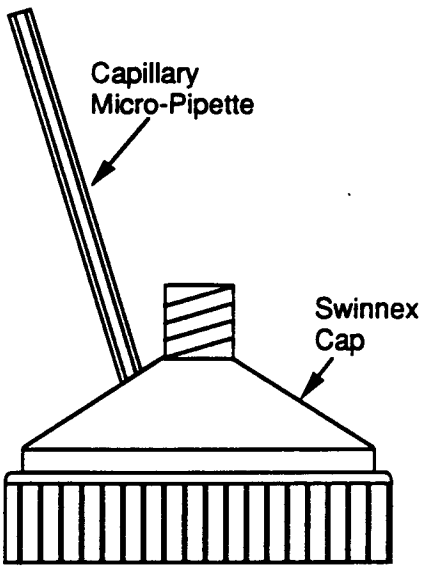
Parts and Equipment List

1. Pipet, micro-sample, disposable, glass, 50 µl	Corning	#7099S
2. Filter holder, top only	Millipore	Swinnex-25
3. Bunsen burner		
4. Needle, 17 gauge		

Instructions

Using a Bunsen burner, heat the needle red hot. Then, penetrate the side of the dome approximately 3 mm from the base of the Luer lock. It may be necessary to penetrate the side several times in the same location in order to accommodate the pipet. Insert a 50 microliter glass micro-pipette into the hole. It must fit tightly in the hole. Do not glue the pipet into the hole since it is easily broken and will need to be replaced on a regular basis.

Schematic



D. Nitrogen Slush Apparatus

Parts and Equipment List

1. Strip-silvered Dewar flask — H. S. Martin, Inc. — #G11400-2265
2. Stopper, 2 hole (to fit flask)
3. Glass Y
4. Vacuum hose
5. Separatory funnel
6. Valve
7. Safety shield
8. Gloves
9. Face Shield

Instructions

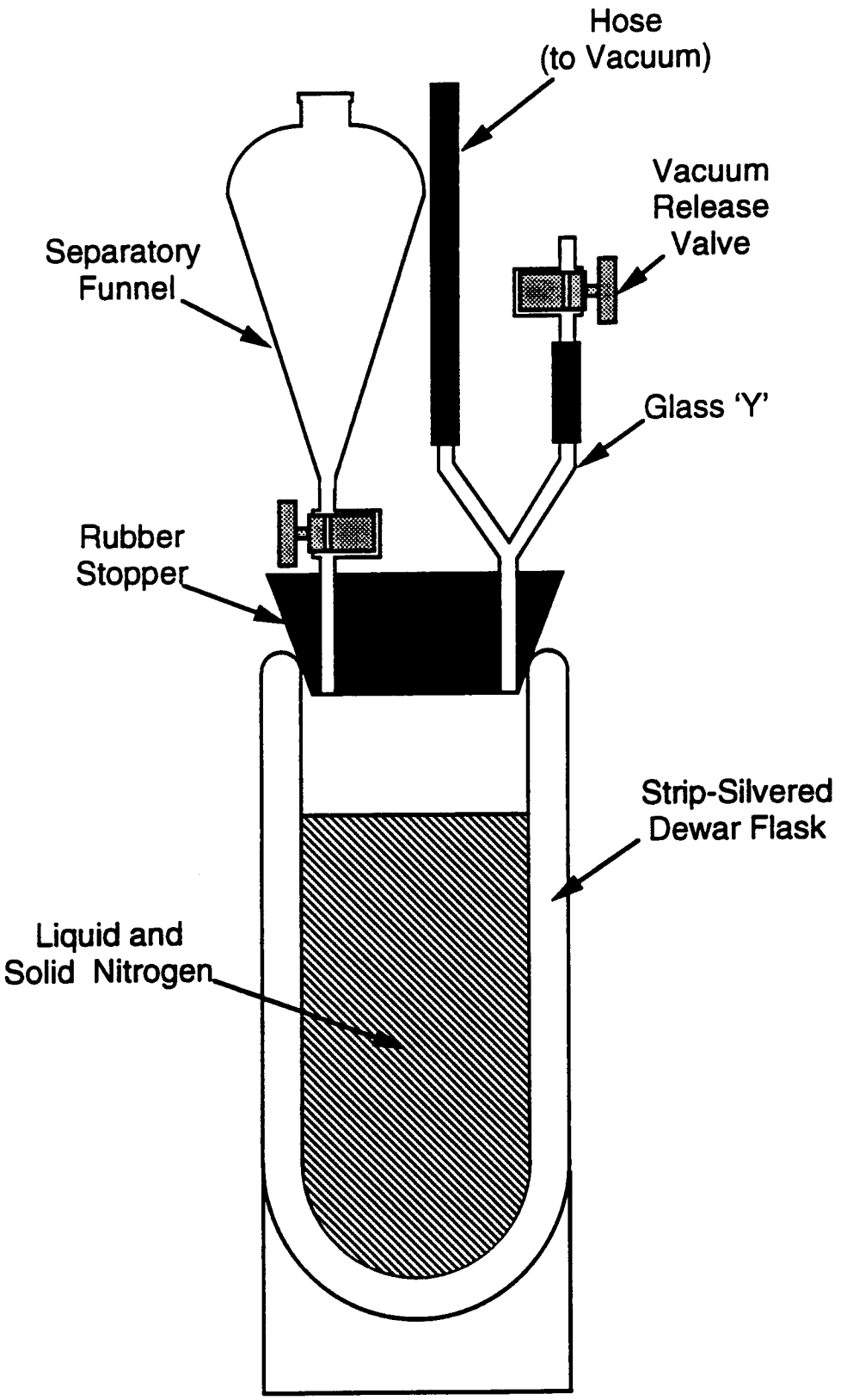
The nitrogen slush is formed by pulling a vacuum on liquid nitrogen. *Because of the possibility of implosion by the Dewar flask or injury due to contact with the cryogenic fluid, appropriate precautions should be taken, including the use of a face shield and gloves, worn by the user, and a safety shield in front of the apparatus. The Dewar should be taped except over the unsilvered strips.*

Assemble the stopper, glass Y, vacuum hose, and separatory funnel as shown in the schematic. Fill the strip-silvered Dewar flask with liquid nitrogen and allow it to come to equilibrium. (Do not apply stopper until this equilibrium is attained — most vacuum systems are incapable of handling the volume of gas generated during this period.) Once the nitrogen has stopped boiling, top off the flask.

Turn on the vacuum and place the stopper on the Dewar. If a tight seal has been formed, the nitrogen will begin to boil vigorously. After 10-20 minutes solid nitrogen should be visible through the unsilvered strip of the Dewar. If there is insufficient nitrogen slush to perform the vitrification step, additional liquid nitrogen may be added by filling the separatory funnel with liquid nitrogen and slowly opening its valve. Once the flask is full, close the valve and allow the liquid to solidify.

The slush is accessed by turning off the vacuum and cracking the vacuum release valve. Once the pressure within the Dewar has equilibrated with its surroundings the stopper is removed. Stir the nitrogen slush with a rod (precooled in liquid nitrogen) in order to break up any lumps of solid nitrogen.

Schematic on Following Page


Hose
(to Vacuum)
Vacuum
Release
Valve
Separatory
Funnel
Glass 'Y'
Rubber
Stopper
Strip-Silvered
Dewar Flask
Liquid and
Solid Nitrogen

E. Humidity Chamber

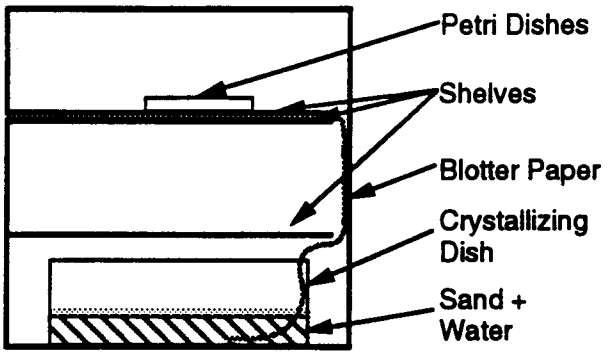
Parts and Equipment List

1. Incubator
2. Plexiglas box with latching door and shelves (12" × 12" × 12")
3. Crystallizing dish, 9" × 3"
4. Blotter paper
5. Sand
6. Double distilled water

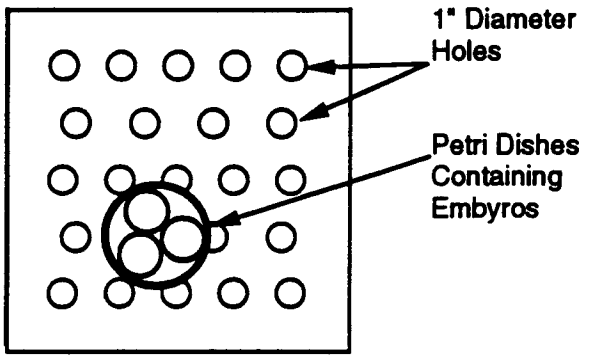
Instructions

We use a 12" × 12" × 12" Lucite box, the door of which is clamped against gaskets on the frame. The box contains a sheet of blotting paper sandwiched between perforated shelves. One end of the blotting paper is immersed in a reservoir of water and sand to keep it saturated. The Lucite chamber is held within a standard bacteriological incubator maintained at 24-25° C.

Schematic



Humidity Chamber (Side View)



Shelf (Top View)