GRADIENT SEPARATION WITH COMPUTER CONTROLLED PUMPS

Abbreviations and codes

Rpm Rotation per minute

IEQ Islet Equivalents

Safety routines

- All work with human material always carries the risk for transferring disease. See Skyddsföreskrift laboratoriearbete KITM AL4731
- Use protective goggles/glasses when running the COBE.
- Hydraulic oil in the COBE contains ethylene glycol (pH >7) and if it leaks out exercise caution. Hydraulic oil is dangerous and if swallowed induce vomiting and contact a doctor. If you get hydraulic oil in your eyes, rinse with water for at least 15 minutes and contact a doctor.
- The used centrifuge set should be thrown in the biological waste containers (*riskavfall*) with liquid absorbing material.

Procedure

Preparation

The COBE centrifuge hydraulic system is checked according to COBE 2991 Blood Cell Processor AL5474 (see Förberedelse inför ö-isolering. Preparation before islet isolation. AL5181) and preparation for gradient separation is performed according to the protocol Förberedelser för gradientseparering med datorstyrda pumpar. Preparation for gradient separation with computer controlled pumps AL5178

Islets are suspended and incubated in about 150 ml 1xUW, see Digerering av pankreas. Pancreas digestion AL5182.

Procedure

In Biological Safety Cabinet

1. Get Heavy Biocoll bags and Light UW-bags for the density gradient from the fridge. Connect the bags to the COBE tubes; green COBE tubing is connected to pump 1 or 3 and to the heavy Biocoll bag, pink tubing is connected to pump 2 or 4 and to the Light UW bag.

Outside the Biological Safety Cabinet

2. Hang density bags on the metal bar over the COBE.
3. Ensure that all colored COBE tubes are clamped. Start pumping the density gradient to the COBE set as follows:

Alternative 1, manual control:

- In PNET press the “Local” button under the pump numbers for pumps to be controlled manually. Pumps 1 or 3 are for the heavy solution; pumps 2 and 4 are for the light solution.

- Prime the light tubing in pumps 2 or 4 first. Remove the clamp on the pink tubing and then press the arrow keys to increase the pump speed. If the pumps do not start, press the “ON/OFF” button.
- Avoid bubbles in the tubing.
- When the solution has reached the tubing manifold press “ON/OFF” to stop the pump. Clamp the pink tubing.
- Pump the heavy density solution via pump 1 or 3. Unclamp the green tubing and set the pump speed to 999. Set a timer for 2 min 10 sec and start the timer when the solution reaches the COBE bag.
- Press “ON/OFF” when the time is up. Clamp the green tubing again.
- All pumps should be paused. While the pumps are paused press the arrow keys so “100” reads on the display panel. This should prevent pump problems associated with the automated portion of the density gradient manufacturing stage.

Alternative 2, computer control:

- Go into the program PNET. For **Pump 2 or 4**, double click the upper field indicating pump speed, another field to enter pump speed should appear.
- Enter a pump speed **700**, remove the clamp on the **pink tubing** and press **Enter**.
- **Immediately double click the same speed field and write in 1**. Press enter when the gradient is at the manifold.
- **Clamp the pink tube** even if the pump does not stop as it could take a couple of seconds for the command to reach the pump. Make sure the pump does not pump backwards, if so, double click the pump speed field, enter 1 and press Enter. It may be necessary to pump more gradient so that the tube looks as it should (not collapsed).
- Pump in the heavy density solution via **pump 1 or 3**. Unclamp the green tubing and enter **999** in the pump speed field.. When the solution reaches the cobe bag start timer for **2 minutes and 10 seconds** and continue pumping.
- Double click the same speed field and write in **1**. When the timer goes off, **stop the pump** by pressing Enter and **clamp the green tubing**. Make sure the pump does not pump backwards, if so, double click the speed field, enter 1 and press Enter. It may be necessary to pump more gradient so that the tube looks as it should (not collapsed).

4. **Open purple tube** to release air in the COBE bag by opening the purple tube (fig. 1). Ensure the COBE speed is at 3000 rpm and **turn superout to 100 ml/min**, push **“Start/spin”**.
5. When full speed is reached push “**Superout**”. Wait until the Biocoll reaches the purple tube and either turn the superout speed to 0 or

immediately press "**Hold**" and clamp the purple tubing at the same time. Stop spinning with "**Stop/reset**". Repeat (steps 4-5) if necessary to remove all bubbles in the tubing.

6. **Turn superout to 0**. Start the COBE again with "**Start/spin**".
7. When full speed is reached, wait another 10 s and then **open green** and **pink tubing**. Ensure the gradient solutions are not being pulled into the COBE. If so, wait a bit longer and continue with this step.
8. On the computer, in the **PNET** program**, click the "Reset and Start all profiles" button** if running both COBE simultaneously. **OR press the "PLAY" buttons** for both pumps simultaneously (or as close as possible)
9. During the time the gradients are pumped into the COBE connect the islet tubing used to load the tissue to the Watson-Marlow 403 pump. In the Biological Safety Cabinet spike the incubation bag and place it on the cold pack on bag hanger. Use the blue tubing for the COBE to the left or the yellow tubing for the COBE to the right.
10. When gradients are loaded (pumps are at 000), clamp the **pink and green tubing** as well as the **main line into the COBE bag**. If the pumps start pumping backwards double click the speed field for that pump, enter 1 and press Enter.
11. Make sure that the heavy and the light gradient has been pumped into the COBE bag by visually examining the bags.

In Biological Safety Cabinet

12. **Open blue or yellow and purple tubing**, make sure the main line is clamped, and start the pump.
13. Pump all the air in the tubing into the purple waste bag and **stop the pump when the islets have reached the manifold** (it is possible to pump at max speed using the gray Watson Marlow 403 pumps). **Clamp the purple tubing**.
14. **Remove the clamp off the main line**.
15. **Pump at a speed of 56% on the gray Whatson-Marlow 403 pump for 3 minutes**.
16. **When the timer goes off, stop the pump and wait for approximately 45 seconds**. Then continue pumping the rest of the tissue.
17. Stop the pump before air enters the tubing and rinse the islet bag by opening the connection to the side bag and load the rest of the tissue. Let the tissue be followed by an air column (About 20cm).
18. **Clamp the blue or yellow tubing and stop the pump** at the time when all the islets, but not the air column, enter into the COBE bag.
19. **Open the purple tubing briefly (<1 sec)** in order to release overpressure and air which may have accumulated at the rotating seal interface. Clamp the purple tubing.

20. Centrifuge for 5 minutes at 3000 rpm.
21. Get the cold T-flasks prefilled with cold wash solution and place them on a freezer tray, 15 flasks for each COBE. In the biological safety cabinet loosen the cap on each to be able to quickly remove the cap.
22. When 5 minutes have passed, station the spike above first flask and **open the yellow/blue tubing** and **then press superout and slowly turn the superout speed to 100 ml/min**. Collect up to 50 ml material in each T-flask, first three and the last flask are empty, others are prefilled. Keep everything as cold as possible during the process.
23. Stop collecting with the **"Stop/reset"** button.
24. Estimate each fractions purity immediately in the microscope, according to pure or unpure. **Keep T-flasks cold. Work quickly. The islets should not be in Biocoll longer than necessary!**
25. **Process the fractions immediately according to the instruction** Bestämning av antalet ö-ekvivalenter. Estimation of tital islet equivalents AL5173

CONTINUE WITH FURTHER COBE RUNS

If there is a third or fourth purification, a modified procedure to speed the set-up process can be used as seen below:

26. Once the COBE on the left has the density gradients loaded and the tubing is clamped it is possible to seal off the tubing of the pink and green lines despite solution in the tubing. Seal the pink and green lines 5 cm from the COBE's manifold.
27. Open a new COBE set and clamp appropriate lines.
28. Use the TCSD II sterile tubing welder to weld the new set to the tubing used previously with the old set. Keep in mind to maintain similar tubing length.
29. This step is to be performed with both pumps in no particular order. Use PNET to reverse flow of the density solution. Double click the speed field and enter "-999", press ENTER.
30. Immediately unclamp the tubing and double click the speed field again, enter "1". Wait until air reaches the density solution bag and press ENTER. Repeat this step for the other pump.
31. Take down the density solution bags from the COBE and place them in the biological safety cabinet. Individually remove the spikes and place them into new density solution bags with the same density as the bag from which they were just removed.
32. Place the new density solution bags onto the metal bar coming out of the top of the COBE and place a temperature pack from the freezer between them.

33. Once the tissue has been collected from the first COBE use the TSCD II tubing welder to weld the islet pump tubing to the new set. **Continue with step 4 above.**

Protocol and Archiving

Protocol is in the database protocol and in the isolation protocol and archived at least 10 years.

Equipment

Apparatus

Centrifuge COBE 2991	MTA 072189, 33876
COBE cooling system	MTA 075061, 078667
Biological Safety Cabinet	MTA 074205
Pump	MTA 34505
Gradient pumps 1, 2, 3, 4	MTA 079354, 079353, 078659, 078660
Sterile welding machine	MTA 079355
Welding machine	MTA 16762

Material

Centrifuge set, Blood cell processing set	746097
Clamps	746199
Tubing, PVC	746216
Flask, black cap 25cm^2 (T-flasks)	746180
Conical, 250ml	746174
Holder for 746180, metal	746302

Reagents

Reagents

Biocoll 1.100 TUNG (Heavy) working solution	767602
Wash solution	767600
Dithizone working solution	767596
UW LÄTT (light) working solution	758240

Overview

Endocrine and exocrine pancreas material can be separated with gradient separation because they have different densities.

Good to know

If there is a problem with the COBE (for example if the COBE does not start or if ”super out” does not work), open the door on the right side of the COBE and

make sure that all the fuses point up. If there is a problem during the loading of the gradients there is a “Stop all” button that stops **all pumps at the same time**. It is possible to continue loading gradients by **pressing the “play” button for each pump individually**. To continue loading gradients for all pumps, even those that have not started, press the “Continue all” button.

References

Related documents

Skyddsföreskrift laboratoriearbete KITM AL4731

COBE 2991 Blood Cell Processor AL5474

Förberedelser för gradientseparering med datorstyrda pumpar. Preparation for gradient separation with computer controlled pumps AL5178

Digerering av pankreas. Pancreas digestion AL5182

Bestämning av antalet ö-ekvivalenter. Estimation of tital islet equivalents AL5173

Förberedelse inför ö-isolering. Preparation before islet isolation. AL5181

Figure 1