

PROCEDURE FOR CRYOPRESERVATION OF CELLS IN BAGS

This procedure is used for cryopreserving large aliquots of cells in cryo storage bags. This procedure is for cell numbers greater than 1-2 billion cells. It is important to follow all biohazard safety precautions when using this procedure. **STERILE TECHNIQUE SHALL BE FOLLOWED AT ALL TIMES USING THIS PROCEDURE**

1. Obtain sample to be cryopreserved. Determine volume, total cell count, and any other pertinent information on the sample.
2. There are two different bags available for cryopreservation of samples. There is a 50 ml and a 250 ml bag size.
3. The 50 ml bag can hold a total volume of 20 ml (including freeze solution). The 250 ml bag can hold a total volume of 72 ml (including freeze solution). The cell aliquots are frozen down in 50% freeze solution and 50% cell suspension. Therefore, the maximum volume of cells for a 50 ml bag is 10 ml and the maximum volume of cells for a 250 ml bag is 36 ml. Exceeding these limits will make storage of the bags very difficult in the storage cassette racking system.
4. When all pertinent information is garnered from the sample then determine if the sample will need to be concentrated down in volume or its volume increased by addition of extra media.
5. To concentrate a large volume of cells down (100 ml or greater) it is best to transfer the cells to an appropriate sized transfer pack.
6. If the cells are in an apheresis bag a transfer pack with coupler can be used to concentrate the cells down. Transfer of the cells is accessed via the apheresis bag port. Moving the cells into a transfer pack will allow the cells to be centrifuged in the Sorvall floor centrifuge.
7. When the cells are in the transfer pack the transfer pack access line can be heat sealed off by using a Sebra heat sealer. The sealed line can be removed from the bag by carefully clipping the annealed portion of the line with a pair of scissors. Care must be taken to not pierce the line area by the bag.
8. When the access line is removed the bag containing the cells can be placed in an overwrap bag and put in one of the Sorvall centrifuge carriers. Balance the bag containing the cells with another "balance bag" containing water.
9. Place the both bags opposite of each other in the Sorvall floor centrifuge. Set the timer to 10 minutes and the speed to 3000 RPM with brake. The cells do not need to be refrigerated during the centrifugation process.
10. When the centrifugation process is complete the cell bag can be carefully removed from the carrier and overwrap bag. Try not to disturb the cell pellet.
11. Place the cell bag in the plasma expresser. To do this you will first need to pull the handle of the plasma expresser fully forward and lock it with the metal catch on the base of the expresser. The bag can be placed on the two hanger pins on the back of the expresser. Use the two holes near the top of the bag for pin placement.
12. Using bench sterile technique, open one of the transfer pack ports and install the coupler end of another transfer pack. This is done by carefully pushing down on the coupler while twisting it. Do not over twist the coupler since that can cause damage to the cell bag.

13. When the coupler is firmly installed then release the handle from the metal catch and let the handle up slowly till it firmly compresses the cell bag. Plasma will begin to flow out of the cell bag into the receiver bag.
14. Allow most of the plasma to be removed from the cell bag. Observe the cell bag to make sure no cells go into the receiver bag. Pressure and plasma flow can be removed from the cell bag by pulling back on the plasma expresser handle. The handle can be placed back in the locked position.
15. Air can be "burped" back into the cell bag by slightly compressing the receiver bag with you one hand. When this is done clamp off the line to the receiver bag. Gently mix the cells in the cell bag by massaging the cell pellet. Check for cell clumps and massage them to bring them back into suspension.
16. The receiver bag can be removed from the cell bag by following step 7.
17. Place the bag containing the cells and the plasma bag in the biological safety cabinet.
18. Plasma transfer lines can be attached to each bag (cell bag and plasma bag). Make sure the roller clamps on the plasma transfer lines are closed.
19. Place the bag containing the cells on a hanger in the biological safety cabinet. Attach a female/female Luer device to the Luer end of the plasma transfer line. A 60 cc syringe can be attached to the open end of the female/female Luer device.
20. With the 60 cc syringe is securely attached, the roller clamp can be opened on the plasma transfer line that is attached to the cell bag. Use the 60 cc syringe as a measuring device to determine the volume of cells in the bag.
21. If any of the cells or cell pellet remains in the bag the empty box from the plasma transfer lines can be used as a cell scraper. This is done by placing the cell bag flat against the inner glass of the biological safety cabinet and using the box end to push the cells down the bag to the bag port area. When this is done the cell bag can be re-hung in the biological safety cabinet and the cells can be syphoned out of the bag into the 60 cc syringe.
22. When all the cells are out of the bag use a tubing stripper to remove any cells from the lines. Strip the lines down to the 60 cc syringe. Push back any air that may have entered into the 60 cc syringe and determine the final volume.
23. Dispense the cells into the cryo bags. Cryo bags have 3 ports and an injection port. Make sure all but one port is closed off when placing the cells in the cryo bag. There are roller clamps on each of the lines for these ports. If the volume of cells is more than the maximum volume allowed in a cryo bag then divide the cells into 2 or more cryo bags. It is best for the cryopreservation process when the cells are divided up evenly between bags.
24. Strip the line that was used to dispense the cells into the cryo bag with a tube stripper.
25. Once the cells are added to the cryo bag(s) then replace the cap on the cryo bag port and make sure the roller clamp on the line that was used is closed.
26. The 60 cc syringe can then be placed on the open end of the female/female Luer device that is attached to the line from the plasma bag. Use the 60 cc syringe to obtain the necessary amount of plasma needed for the freeze solution. (see freeze solution procedure for determining volumes needed)

27. The freeze solution can be made in a bag or 50 ml Falcon tube. Total volume of the freeze solution will determine which is used. (see freeze solution procedure for how to make freeze solution in either a bag or a tube)
28. With the freeze solution made using the freeze solution procedure the bag of freeze solution can be placed in the refrigerator to chill (usually 10-15 minutes is sufficient).
29. When the freeze solution is adequately chilled a plasma transfer line can be added to the freeze solution bag. A female/female Luer device can then be attached to the male Luer end of the plasma transfer line. This will allow the use of an adequately sized syringe to be attached and used to transfer the freeze solution to the cryo bag.
30. With the syringe pull an equal volume of freeze solution, this will be added to the cells in the cryo bag using one of the ports to the bag.
31. When the syringe attached to the cryo bag port open the roller clamp to the port. Carefully but quickly inject the freeze solution into the cryo bag while mixing the cells and freeze solution in the cryo bag. The mixing process can be accomplished by shaking the bag up and down with one hand.
32. When all the freeze solution had been injected from the syringe. Remove the syringe from the bag port. Allow a little air to enter. This will allow the remaining freeze solution in the line to drain down into the cryo bag. Attach the syringe back onto the cryo bag port. Mix the bag once again.
33. All of the air in the cryo bag needs to be removed at this time. Use the attached syringe to remove the air. Air bubbles in the port area will need to be squeezed out and guided out to the syringe. Any air in the bag may cause false readings with the Cryomed probe during the cryopreservation process.
34. Air bubbles can and do create an unsupported area of the bag. If the bag does get bumped where an air bubble is located the bag plastic could fracture. It is imperative that all bubbles are removed from the bag.
35. When it has been determined that all the air bubbles are removed then the roller clamp can be closed on the cryo bag and the line cap can be placed back on. Save the syringe if there are more bags from the same donor to freeze since it can be reused for them.
36. The main line coming out of the cryo bag will need to be removed. This line is between two yellow port caps. This line can be removed by using a Sebra heat sealer to seal the line. The line will need to be sealed below the yellow cap ports and in the same plane as the ports. The tubing used for this central line is not able to withstand cryogenic temperatures and must be protected against bumps. The large yellow port caps can accomplish this protection.
37. Complete a bag label with the appropriate information on it. This label slips into the pocket provided on the cryo bag. A heat seal using the Sebra heat sealer is done in the middle of the pocket entrance. This is done to hold the label in the pocket.
38. Repeat steps 30-37 for any addition cryo bags that need to have freeze solution added.
39. When all the bags are done they can be cryopreserved in the Cryomed controlled rate freezer. (see Cryomed freezer instructions for using this device)

MATERIALS NEEDED FOR THIS PROCEDURE

- Cryo bags
- Female/female Luer device
- Sebra heat sealer
- Syringes
- Needles
- Sorvall floor centrifuge
- Plasma expresser
- Prepared freeze solution
- Plasma transfer lines
- Transfer packs
- 50 ml Falcon tubes