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Subculture of Normal Human Cells

1. Allow all three solutions in the DetachKit (HepesBSS, Trypsin, TNS) to reach room temperature and remove the seals from the bottles. Cold solutions will not work properly.

2. Fill the new culture containers with fresh medium in a laminar flow cabinet. PromoCell recommends 200 µl medium / cm² of culture dish bottom.

For general tissue culture dishes:

Lid diameter	35 mm	60 mm	100 mm
Tissue culture dish base	9.6 cm²	20.4 cm²	57 cm²
200 µl medium / cm² corresponds to approx.	2 ml	4 ml	11 ml

For general tissue culture flasks:

Tissue culture dish base	25 cm²	75 cm²
200 µl medium / cm² corresponds to approx.	5 ml	15 ml

3. Incubate the culture containers in an incubator at 37°C, 5 % (V/V) CO_2 and steam saturated atmosphere for at least 30 minutes. Make sure that the screw lids on the culture flasks are only lightly closed so that a gas exchange is possible through the gap between lid and flask.

4. Examine the cell culture under the microscope. The monolayer should be sub-confluent (60 - 80 % confluence) and many mitotic figures should be visible.

5. Open the cell culture flask carefully in a laminar flow cabinet and remove the medium using a sterile pipette. Do not touch the cell monolayer with the pipette. Replace the medium with 5 ml HepesBSS and wash the cells for about 30 seconds by gently swirling the culture flask.

6. Remove the HepesBSS and cover the cell monolayer with about 100 µl trypsin / EDTA solution / cm² of culture container surface. Immediately afterwards tighten the cap and examine the cells under the microscope. Observe the separation of the cells under the microscope.

7. When approximately 50 % of the cells are loose, gently tap the side of the culture container to loosen the remainder of the cells. If the cells are not completely separated, trypsinizate a further minute. Warming the culture at 37°C in an incubator will speed up the separation and will not damage the cells. The total trypsinization time should not exceed 7 minutes.

Note: Over-trypsinization causes irreversible damage!

8. When the cells are completely detached, add about 100 µl Trypsin Neutralising Solution / cm² of culture container surface and remove the cell-suspension with a sterile pipette. Place the cell suspension in a centrifuge tube.

9. Centrifuge the cells at about 220 x g for 4 minutes at room temperature (cold temperatures will cause irreversible damage!) and remove the clear upper layer. Check that the cells are all at the bottom of the tube. The upper layer must be completely clear.

Note: As the cell cluster is often only lightly attached to the bottom , it's easily removed. **Be careful !**

10. Add about 1.5 ml medium and re-suspend the cells by drawing cells and medium slowly and carefully up and down with a sterile pipette tip. Make up with medium to the 2 ml level exactly and then take a precise amount of the suspension to determine the number of the cells.

11. Distribute the cell suspension in the usual way and in the required density to the culture containers which have been prepared following point 2 and 3 of these instructions. If you haven't used a PromoCell medium for the re-suspensation, please make sure that the added seeding medium in the prepared culture containers doesn't amount to more than 2 % (V/V) of the medium used for the re-suspensation.

12. Place the seeded cell culture containers in an incubator at 37°C, humidified and 5 % (V/V) CO_2. Close the screw lids on the culture containers by half a turn to allow gas exchanges to take place.

13. Examine the cells microscopically after 24 hours. At least 80 % of the cells should adhere. Some cells will be swimming in the medium or only lightly attached while most of the cells should be spread out on the bottom of the container. In this stage most of the cells will be growing alone or in small clusters.
Once the cells have adhered (after minimum 24 hours) remove the medium with a pipette and replace it with the same volume of fresh, warmed medium.

14. Incubate for a further 24 hours, Then examine under the microscope. After this period the culture should have mitotic clusters which shows that the cells are in a proliferative state.

15. Replace the medium every two days after this point.

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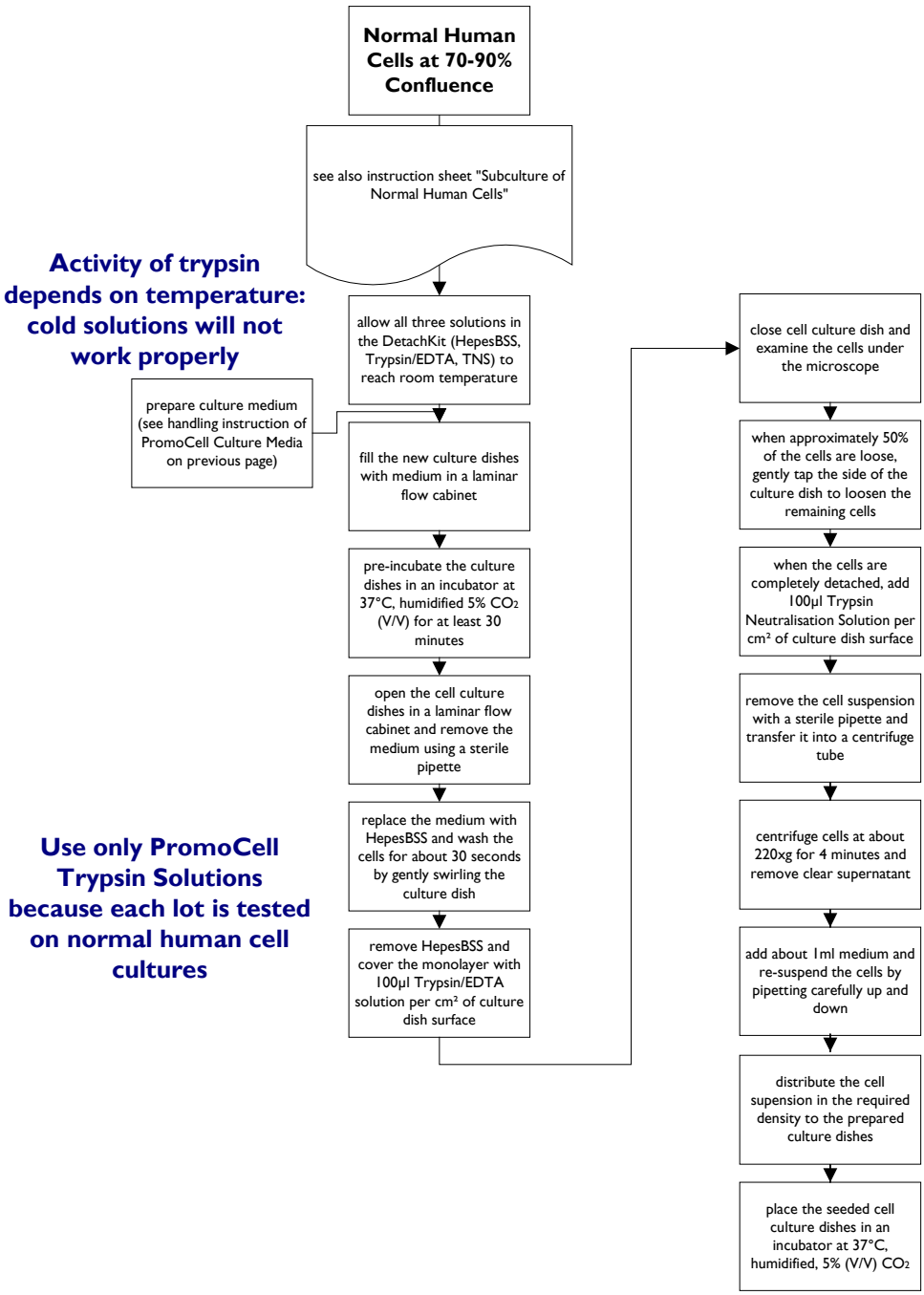

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