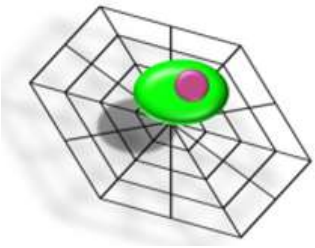


Circulating Tumor Cells TheRapeutic Apheresis: a novel biotechnology enabling personalized therapy for all cancer patients

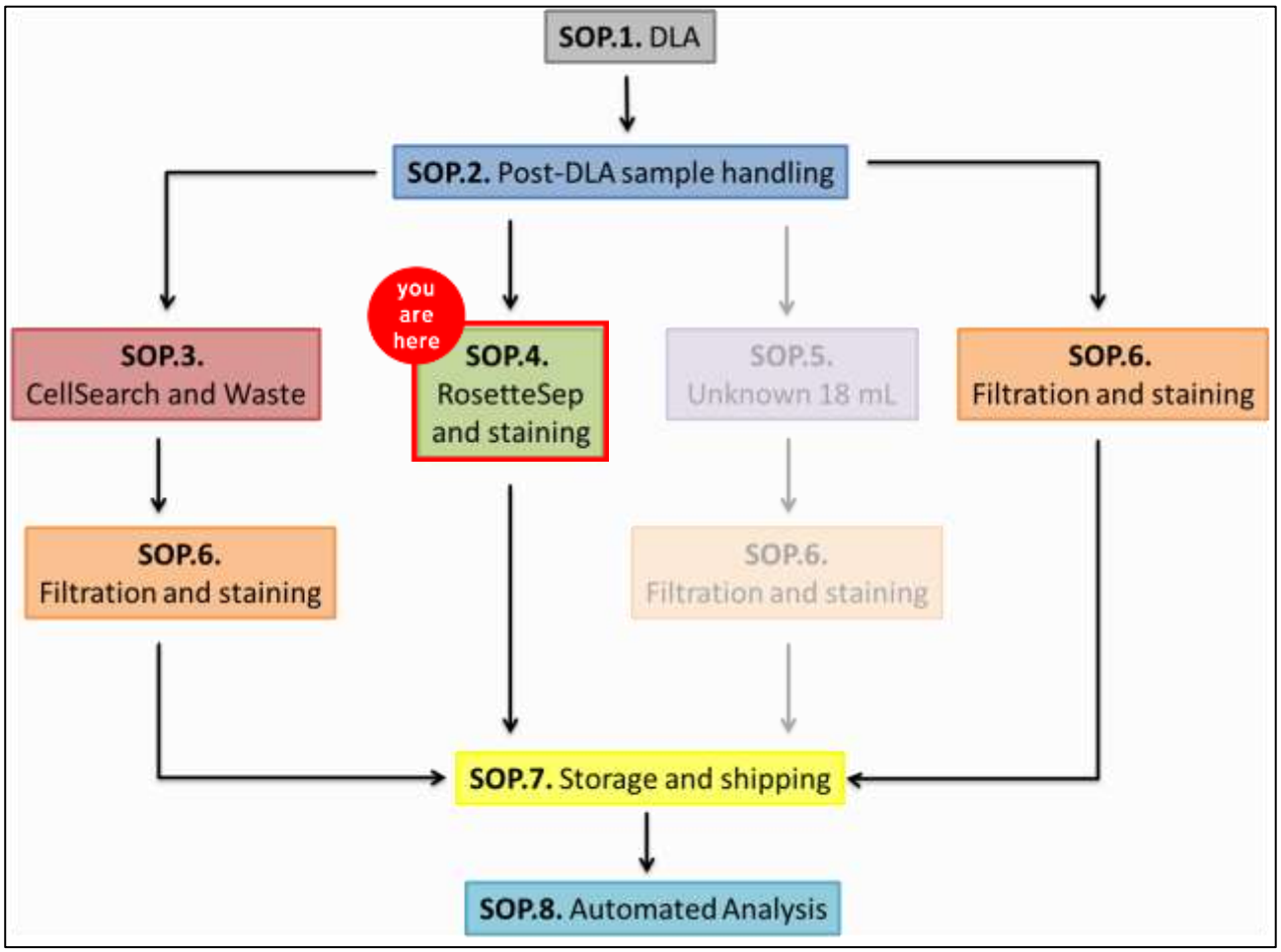
SOP.4. RosetteSep and staining Of DLA samples

Introduction

This Standard Operating Procedure (SOP) describes the depletion of leukocytes from DLA samples, thereby enriching the CTC, followed by subsequent filtration and staining. Note that this staining procedure resembles the staining procedure from **SOP.6.**, with one critical difference. This SOP is followed by **SOP.7**.

This is SOP.4. RosetteSep and staining; version 1.0-092015

Workflow of procedures in the CTCTrap program



SOP.4. RosetteSep and staining

Of DLA samples

The sample used for this SOP is 18 mL of diagnostic leukapheresis product (DLA), which is Cellsave fixed overnight. Also, 2x 10 mL EDTA blood tubes is necessary to perform the RosetteSep protocol.

After RosetteSep, the sample can be filtered with the VyCAP system and stained and scanned.

The complete procedure is described in this SOP.

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1. Buffers and materials

1.1 Rosette Sep

- RosetteSep™ CTC Enrichment Cocktail Containing Anti-CD36 (Stemcell Technologies Catalog# 15167)
- Ficoll-Paque PLUS (GE Healthcare, Product code:17-1440-02)
- Phosphate buffered saline (PBS) with 2% fetal bovine serum (PBS/2% FBS) (store at 4°C)
- 1xPBS
- 50mL tubes
- Centrifuge
- Hematology analyzer (e.g. Coulter Counter)

1.2 Filtration and staining

- Filtration station (VyCAP)
- FIltertube with sieve (VyCAP)
- Sieve standard including sponge (VyCAP)
- PBS/BSA 1%
- PBS/BSA 1%/saponin 0.05% for staining (make fresh once per week)
- Formaldehyde 1% in PBS for fixing

Staining mix:

  - Anti-CD45-PerCP (*Life Technologies, MHCD4531*, clone HI30): final concentration 4ug/mL (2 µL from stock solution when stock solution is 100 µg/mL)
  - Anti-CKpan-NanoParticles 575 (*AcZon*, clone C11 and AE1/AE3): final concentration 3,5ug/mL (1 µl from stock solution is 3.5 µg/mL)

Adjust to a total volume of 50 uL with PBS/BSA 1%/saponin 0.05% (47 µL).

- Mounting medium (20 µL per microsieve): ProLong® Diamond Antifade Mountant with DAPI (*Life Technologies, P36971*).
- Coverslip: 2x 0.85 cm^2 custom cut, thickness #1 (0.13-0.16mm) (Menzel-Gläser, Saarbrückener, Germany).

2. RosetteSep

1. Measure the white blood cell concentration (WBC) in the DLA product using a hematology analyzer.
2. If WBC concentration of DLA is higher than $70x10^6$cells/mL, dilute the DLA to $70x10^6$ WBC/mL using PBS/2%FBS and continue the protocol with 18mL of this diluted DLA product.
3. Concentrate the red blood cells (RBC) in the EDTA tubes by centrifuging the two EDTA tubes for 8 minutes at 800xg.
4. Remove the plasma and buffy coat. Mix the tube with only RBC by tilting the tube several times. Pool the RBC from both tubes in one tube.
5. Measure the RBC concentration of the concentrated RBCs using hematology analyzer. If concentration is too high for accurate measurement, dilute 1:1 using 1xPBS.
6. The WBC to RBC ratio should be 1:40. Calculate the amount of RBC that need to be added to the DLA using the following calculations:

$$18\ \text{mL} \cdot (\text{WBC Concentration}) = \text{X}$$

$$40 \cdot \text{X} = \text{Y}$$

$$\frac{\text{Y}}{(\text{RBC Concentration})} = \text{mL RBC to be added}$$

7. Add the calculated amount of RBC to 18mL DLA.
8. Measure the WBC and RBC concentration of this mixture. RBC to WBC ration should be at least 40:1. To confirm: $\frac{\text{RBC Concentration}}{\text{WBC Concentration}} => 40$
9. Add 50uL of RosetteSep™ CTC Enrichment Cocktail Containing Anti-CD36 reagent for each 1 mL of sample. E.g. 18 mL DLA + 3 mL RBC = 21mL in total; add 1050 µL of reagent (Figure 1, step 1).
10. Mix well by vortexing.
11. Incubate 20 minutes at room temperature (Figure 1, step 2).
12. After incubation, dilute the sample with an equal volume of PBS/2%FBS and mix gently.
13. Prepare two 50mL tubes with each containing 15 mL of Ficoll-Paque PLUS as a density gradient.

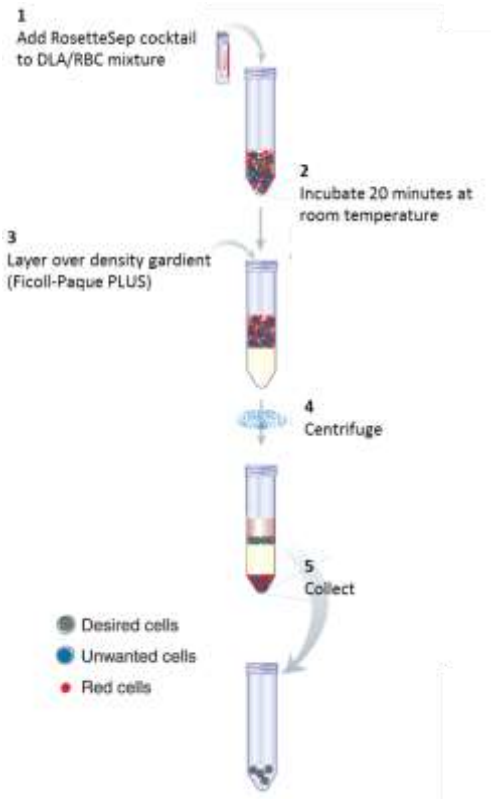

Figure 1 Schematic workflow of the RosetteSep in which the antibody is added to the sample (step 1), incubated (step 2) and separated by density gradient (step 3, 4 and 5).

14. Divide the sample over the two tubes by layering very carefully ½ part of the sample over the density gradient in each 50mL tube (see Figure 1, step 3). Note: Be careful to minimize mixing of the density gradient medium and sample.

15. Centrifuge for 20 minutes at 1200xg at room temperature with the brake off (see Figure 1, step 4).
16. Collect the enriched cells from the density gradient medium - plasma interface. Pipet the collect cells into a 50mL tube (see Figure 1, step 5). To make collection more easy, first remove half of the fluid on top of the desired cells by pipetting and discarding, then continue with collection of desired cells with a small pipet . Note: Sometimes it is difficult to see the cells at the interface. It is advisable to remove some of the density gradient medium along with the enriched cells in order to ensure their complete recovery.
17. Wash the cells by adding 2 volumes of PBS/2%FBS to the volume of the collected cells.
18. Centrifuge for 8 minutes at 300xg at room temperature.
19. Remove the supernatant. Do not disturb the cells and leave about 1000 µL on top of the cells.
20. Resuspend the cells in a total volume of 9 mL 1xPBS.

3. Filtration

3.1. General notes

- During incubation, make sure the microsieve does not touch the sponge or any other surface to prevent drying of the microsieve.
- It is advised to use clean cover slips for optimal analysis. Wash each cover slip with EtOH and dry by blowing air.

21. Connect filtration tube containing the sieve onto the filtration station (Figure 2).
22. Check before filtering if the pressure is correct. The sieve has to be in the filter holder and the lid of the tube has to be closed. When the pressure is in the correct range, a green light will show.
23. Pressure is OFF: apply the sample.
24. Put the pressure ON, and keep on to filter the complete sample, but do not let the sieve run dry.
25. Shut the pressure OFF before the sieve runs dry.
26. Remove the microsieve carefully from the filtration station.
27. Place the sieve in a sieve standard (Figure 3) and remove any remaining solution on the sieve by gently pressing down the sieve down on the sponge.
28. Wash the sieve once with 50 µL PBS/BSA1%.
29. Apply 50 µL of staining solution and incubate 15 min at 37°C in a humidified environment (like a cell culture incubator or hotplate).
30. Remove the solution by gently pushing the sieve down.
31. Wash the sieve once with 50 µL PBS/BSA1%.

Figure 2 Complete filter tube and sieve in the filtration station.

Figure 3 Microsieve in sieve standard.

32. Apply 50 µL PBS/BSA1% and incubate 5 minutes at RT to remove unbound antibodies.

33. Remove the solution by gently pushing the microsieve down.

34. Fix the cells with 50 µL 1% formaldehyde for 10 min at RT.

35. Remove the solution by gently pushing the sieve down.

36. Wash the microsieve two times with 50 µL PBS/BSA1%.

37. Turn the sieve upside down in the staining holder. Pipet 20 µL ProLong® mounting medium. By moving your pipet on the edge of every lane, the lanes will fill with mounting medium (this requires in total approximately 10 µL medium). This filling of the lanes is visible by eye, but takes a little patience and some practice. Apply the coverslip on the backside of the microsieve.

38. Turn the sieve back in the upright position. Apply the remaining mounting medium gently on the microsieve. Apply the coverslip and prevent any air bubbles.

39. Analyse the samples with the fluorescence microscope as soon as possible. Store the samples at minus 20°C for storage, distribution or future molecular analysis.

4. Scanning

40. Use the next filter cubes for automatic scanning of the microsieves on a mercury arc fluorescence microscope (see *Optical Filters* document for more information) (see Table below)

41. Use a 20X objective, with minimal 0.45NA.

42. Define the optimal exposure time for the scanning of the microsieves. Use this exposure time for every sample. Make sure the lamp is not too old and beware of bleaching the sample.

43. Label the slide with patient number, site of processing, date and other useful information.

44. Store the microsieve at -20°C after scanning (continue in **SOP.7. and SOP.8**).

Filter cube	*Excitation (nm)*	*Dichroic (nm)*	*Emission (nm)*
DAPI	377/50	409 LP	409 LP
PE	543/22	562 LP	593/40
PerCP	435/40	510 LP	676/29

5. Checklist SOP.4.

Sample name	
Operator name	
Draw date Clinical site	
Prep date Clinical site	
Microsieve scan date Clinical site	
WBC count in DLA sample	
Diluted WBC with PBS/2%FBS?	☐ Yes ☐ No
Concentrated pooled RBC count in EDTA tubes	
Diluted RBC with PBS?	☐ Yes ☐ No
$18\ \text{mL x (WBC Concentration)} = \text{X}$ $40\ \text{x X} = \text{Y}$ $\frac{\text{Y}}{\text{(RBC Concentration)}} = \text{mL RBC to be added}$	X = Y = mL RBC to be added =
WBC count in total sample (step 8)	
RBC count in total sample (step 8)	
$\frac{\text{RBC Concentration}}{\text{WBC Concentration}}$	=
Total volume of RosetteSep cocktail added	

Volume enriched cells (step 16)	
Was filtration performed at -100mbar (shows green light)?	☐ Yes ☐ No ☐ I don't know
Total filtration time	_____ min: _____ sec
Was staining performed for 15 min at 37°C?	☐ Yes ☐ No
Was the staining mix as described in this procedure used?	☐ Yes ☐ No
Was the washing step of 5 min performed?	☐ Yes ☐ No
Was the sample fixed with 1% formaldehyde for 10 min?	☐ Yes ☐ No
Was the coverslip mounted on both sides of the microsieves?	☐ Yes ☐ No

What are the excitation/emission values from the cubes used for scanning?
DAPI

PE

PerCP

Objective	Magnification:	NA:

Was the sieve stored at (at least) -20°C?	☐ Yes ☐ No

Notes: