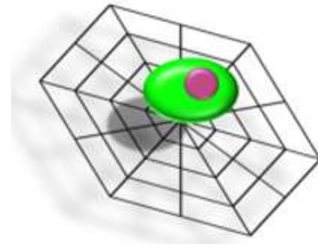


Circulating Tumor Cells TheRapeutic APheresis: a novel biotechnology enabling personalized therapy for all cancer patients

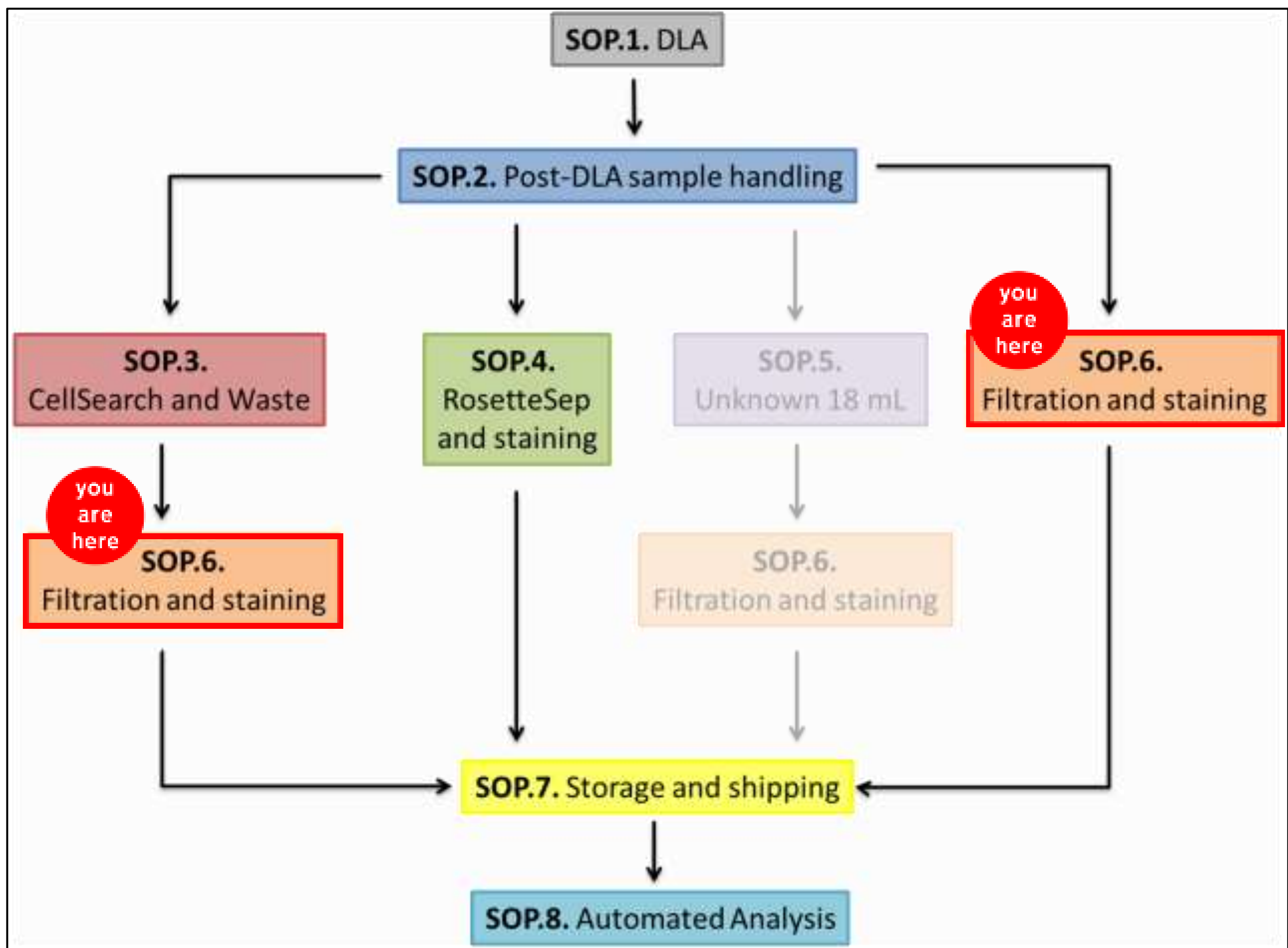
SOP.6. Filtration, Staining and Scanning Of cells on microsieves

Introduction

This Standard Operating Procedure (SOP) describes the filtration, staining and scanning of CTC which are captured on a microsieve. The sample used for filtration can be Waste from the CellSearch (from a whole blood sample or a DLA product) (**SOP.3.**), eluate from the CTCTrap (**SOP.5.**) or a DLA product (**SOP.2.**). This SOP will be followed by **SOP.7.**, for the storage of microsieves and cartridges, and **SOP.8.,** describing the analysis of the images of the scanned microsieves with ICY.

This is SOP.6. Filtration, Staining and Scanning; version 1.0-092015

Workflow of procedures in the CTCTrap program



SOP.6. Filtration, Staining and Scanning Of cells on microsieves

This SOP.6. describes the details of the filtration with the VyCAP filters and use of the microsieves, as well as the defined staining conditions and scanning settings.

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1. Buffers

All buffers should be filtered before use. Keep them as much closed as possible to prevent any contamination by dirt or dust particles.

- PBS/BSA 1% for washing
- PBS/BSA 1%/saponin 0.15% for permeabilization (make fresh once per week)
- PBS/BSA 1%/saponin 0.05% for staining (make fresh once per week)
- Formaldehyde 1% in PBS for fixing
- ProLong® Diamond Antifade with DAPI mounting medium for analysis and storing

2. Staining solution

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

Add to 50 µL total volume 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).

3. Filtration station

See *Figure 1*. 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.

Figure 1 Complete filter tube and sieve in the filtration station. Correct filtration pressure shows a green light in the corner.

4. Filtering

1. Note the total volume of the sample. The eluate tube during filtration can contain up to 45 mL.
2. Pressure is OFF: apply the sample. See *Figure 2A*.
3. Put the pressure ON, and keep on to filter the complete sample, but do not let the sieve run dry. See *Figure 2B*. Filter for a maximum time of 10 minutes, if the complete volume was not filtered, see 4.1 on how to proceed.
4. Shut the pressure OFF before the sieve runs dry.
5. Remove the microsieve carefully from the filtration station.

4.1. Clogging during filtration

When a sample contains a lot of white blood cells, it can happen that all the pores of the microsieve are filled before the complete sample is processed. This sample clogs and the volume is passed very slowly without actual filtering. In case of clogging during filtration, proceed as follows:

4a. Filter for a maximum time of 10 minutes, after which the pressure can be switched OFF.

4b. Transfer the remaining, unfiltered sample to a tube with volume markings (this can be the original tube).

4c. Note the volumes which was not filtered and subsequently filtered; these can be used to calculate how much whole blood volume was used for filtration.

4d. Discard the remaining, unfiltered sample.

5. Remove the sieve carefully from the filtration station.

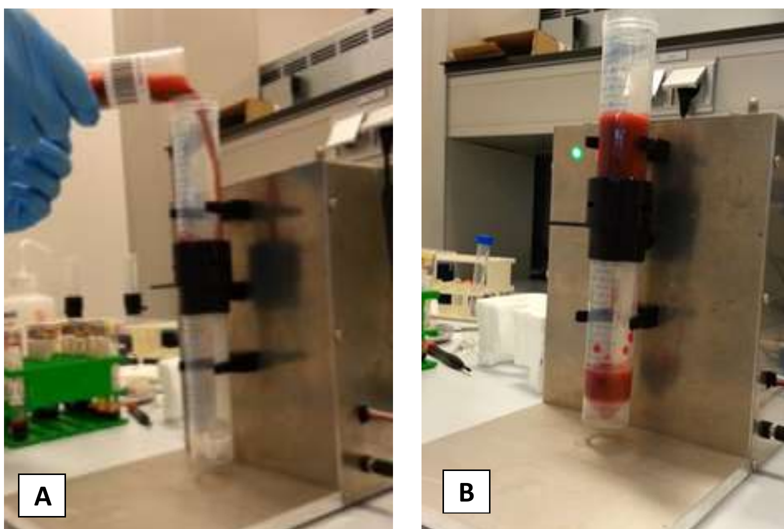

Figure 2 Filtering a sample in the filtration station. A sample is applied directly on the sieve in the filter tube (A). Filtration of the sample with the correct pressure can take up to 10 minutes, depending on the amount of white blood cells present in the sample (B).

5. Staining

5.1. General notes

- During incubation, make sure the microsieve does not touch the sponge or any other surface to prevent drying of the microsieve.
- (*) At this point the protocol can be stopped and the microsieve can be placed at 4°C with some PBS/BSA1% solution on top. Make sure the microsieve does not touch any surface or that the microsieve dries.
- (**) At this point the protocol can be stopped and the microsieve can be placed in the fridge with some PBS/BSA1% solution on top. However, it is best if the mounting medium and cover slip are immediately placed and the sample is stored at -20°C.
- It is advised to use clean cover slips for optimal analysis. Wash each cover slip with EtOH and dry by blowing air.

See Figure 3 for reference.

6. Place the microsieve in a sieve standard and remove any remaining sample on top of the microsieve by gently pressing down the sieve down on the sponge (*).
7. Wash the sieve once with 50 µL PBS/BSA 1%/saponin 0.15%.
8. Apply 50 µL PBS/BSA 1%/saponin 0.15% and incubate 15 min at RT for permeabilization.
9. Remove the solution by gently pushing the sieve down.
10. Apply 50 µL of staining solution and incubate 15 min at 37°C in a humidified environment (like a cell culture incubator or hotplate).
11. Remove the solution by gently pushing the sieve down.
12. Wash the sieve once with 50 µL PBS/BSA 1%.
13. Apply 50 µL PBS/BSA 1% and incubate 5 minutes at RT to remove unbound antibodies.
14. Remove the solution by gently pushing the microsieve down.
15. Fix the cells with 50 µL 1% formaldehyde/PBS for 10 min at RT.
16. Remove the solution by gently pushing the sieve down (**).
17. Wash the microsieve two times with 50 µL PBS/BSA 1%.
18. 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.

19. 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.
20. Scan the samples with the fluorescence microscope as soon as possible. Store the samples at minus 20°C for storage, distribution or future molecular analysis.

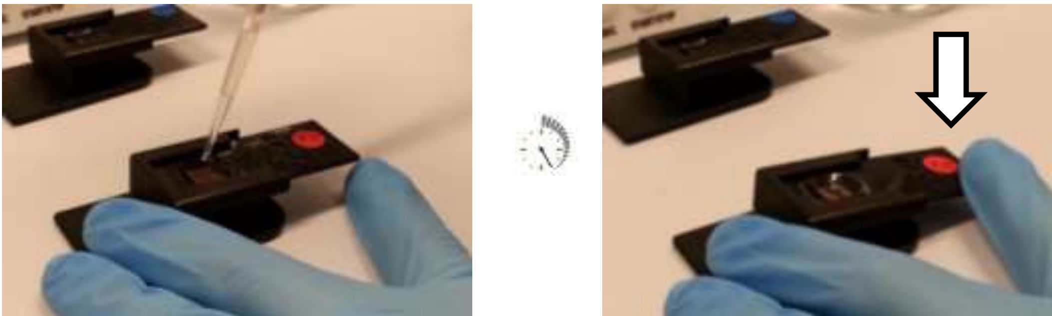

Figure 3 Washing, permeabilization, staining and fixation of the cells are all performed on the sieve in the sieve standard. Solutions can be applied directly on the sieve (left). The solution will stay on the sieve during incubation. By gently pushing down the end of the slide, the sieve will come in contact with the sponge underneath and this will absorb the solution (right).

6. Scanning

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

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

22. Use a 20X objective, with minimal 0.45NA.
23. 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.
24. Label the slide with patient number, site of processing, date and other useful information.
25. Store the microsieve at -20°C after scanning (continue in **SOP.8**).

7. Checklist SOP.6

Sample name	
Operator name	
Draw date	Clinical site
Prep date	Clinical site
Microsieve scan date	Clinical site
Total sample volume	
Was filtration performed at -100mbar (shows green light)?	☐ Yes ☐ No ☐ I don't know
Total filtration time	_______ min: _______ sec
Volume not filtered after 10 minutes (clogging of microsieve)	
Volume filtered after 10 minutes	
Was permeabilization performed for 15 min with 0.15% saponin?	☐ Yes ☐ No
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

<table>
<tr><td colspan="2">Was the coverslip mounted on both sides of the microsieves?</td><td>☐ Yes ☐ No</td></tr>
<tr><td colspan="3">What are the excitation/emission values from the cubes used for scanning?
DAPI

PE

PerCP</td></tr>
<tr><td>Objective</td><td colspan="2">Magnification: NA:</td></tr>
<tr><td colspan="2">Was the sieve stored at (at least) -20°C?</td><td>☐ Yes ☐ No</td></tr>
<tr><td colspan="3">Notes:</td></tr>
</table>