VyCAP

Filtration and staining protocol

Optimized for whole blood samples

Version 2.4

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Reagents

1. Blood collection tubes:
   - Circulating Tumour Cell TransFix/EDTA Vacuum Blood Collection Tubes, Cytomark prod code CTC-TVT-09
2. Filtered PBS/BSA 1%.
3. FIX&PERM, Nordic MUbio, product code GAS-002M or GAS-002. Only small amounts are needed and GAS-002M is mostly sufficient.
4. ProLong® Diamond Antifade with DAPI mounting medium (Thermo Fisher, P36962)
5. Immunofluorescence labels for CTC detection:
   - Anti-Pan Cytokeratin CK-11 PE (Cell Signaling Technology, #5075S, clone C-11)
   - Anti-Pan Cytokeratin AE1/AE3 eFluor 570 (eBioscience, 41-9003-82, clone AE1/AE3)
   - Anti-CD45 APC (eBioscience, 17-0459-41 / 42, clone HI30)
   - Anti-CD16 APC (BioLegend, #302011, clone 3G8)

Filtration

The blood collected in the Transfix (CTC-TVT-09) tube needs to be filtered between 24 and 48 hrs after the draw.

1. Set the pressure to 200 mbar.

   ***NOTE* : In general the pressure setting is correct if the blood passes the filter with a flow rate of approximately 1-3 ml/min.**
   Besides the age of the blood sample the flow rate is donor dependent and will differ from sample to sample.

2. Check the volume of the sample. The eluate (waste) tube of the filtration unit can maximum hold a volume 45mL.
3. Insert a new filtration unit on the pump-unit.
4. Switch ON the pump.
5. Transfer the whole blood sample to the sample side of the filtration unit.
6. Switch OFF the pump as soon as the whole sample has passed. Don't leave the pump-unit switched ON after the sample has passed.
7. To wash away the remaining blood components, add 1 ml of PBS + BSA 1% to the sample side of disposable unit
8. Switch ON the pump and switch it OFF when the PBS passed the filter.
9. Repeat step 7 and 8.
10. Remove the slide with the sieve from the disposable filtration holder and transfer it to the staining holder.

Figure 1: Filtration components.

Staining procedure for microsieves

1. Place the slide with microsieve in the staining holder.
2. Remove any remaining blood and PBS on the sieve by gently pressing down the slide and sieve on the absorber. In very few cases the absorber is unable to remove the excess blood from the sieve by pushing on the absorber. In that case you can use a tip of a tissue to remove the excess by putting the tip of tissue in one of the corners of the sieve surface.

   The absorber should be replaced for every new sieve.

   ***NOTE* : It is very important to avoid drying of the microsieve during the staining procedure. As soon as the washing buffer or staining reagents are removed immediately proceed with the next step. Drying of the sample will result in bad morphology and fluorescence labelling.**

3. Release the pressure on the slide (no contact between sieve and absorber anymore)
4. Wash the sieve, by adding 75 µL PBS + BSA 1% onto the sieve and next push the slide down on the absorber again. This will remove the PBS+BSA 1% wash buffer.
5. Release the pressure on the slide (no contact between sieve and absorber anymore)
6. Repeat step 4 and 5.

Fixation:

7. Add 100µL of Component A, of the FIX&PERM reagents gently onto the microsieve.
8. Incubate 20 minutes at RT
9. Remove the fixation solution by gently pushing the sieve onto the absorber.
10. Wash the sieve, by adding 75 µL PBS+BSA 1% onto the sieve and next push the slide down on the absorber again. This will remove the washing buffer

Permeabilization and staining:

11. Prepare the permeabilization/staining cocktail. Take 50µL of component B of the FIX&PERM reagents. Table below displays the volumes of each of the immunofluorescence labels that needs to be added to the 50µl of component B.

Component	Volumes (µL)
Anti-Pan Cytokeratin CK-11 PE (Cell Signaling Technology, #5075S, clone C-11)	0.5

Anti-Pan Cytokeratin AE1/AE3 eFluor 570 (eBioscience, 41-9003-82, clone AE1/AE3)	0.5
Anti-CD45 APC (eBioscience, 17-0459-41, clone HI30)	1.0
Anti-CD16 APC (BioLegend, #302011, clone 3G8)	1.0

Gently transfer the whole 53µL permeabilization/staining cocktail onto the microsieve. Add the reagents gentle and make sure the whole sieve surface is covered.

12. Incubate for 20 minutes at RT.
13. Remove the staining solution by gently pushing the microsieve down onto the absorber
14. Wash the sieve, by adding 75 µL PBS+BSA 1% onto the sieve and next push the slide down on the absorber again. This will remove the washing buffer
15. Repeat step 13
16. Add another 75µL PBS/BSA 1% and incubate 5 minutes at RT to remove unbound antibodies.
17. Remove the solution by gently pushing the microsieve down onto the absorber. In this step remove as much fluid as possible by keeping the sieve longer in contact by pushing it onto the absorber. The sieve surface looks dry after completing this step.
18. Let the sieve dry more for 10 minutes at room temperature.
19. Remove the slide from the staining holder and insert it again upside down (the backside of the sieve is now facing the user.
20. Add 15µL of the mounting medium onto the backside of the microsieve in the centre. The back side of the microsieve contains little grooves which need to be filled with mounting medium to maintain maximum signal after storage of the slide. Distribute the mounting medium with the pipette tip. After the grooves are filled place a VyCAP cover glass (prod # CG-10) onto the backside.
21. Remove the slide from the staining holder and insert it again upside down (the front side of the microsieve is now facing towards the user again).
22. Add mounting medium to the front side of the microsieve by adding a small droplet of 5-6 µL in the centre of the microsieve at the position of the cross.
23. Place a VyCAP cover glass (prod # CG-10) onto the front side.
24. Incubate 10 minutes .
25. Acquire images.

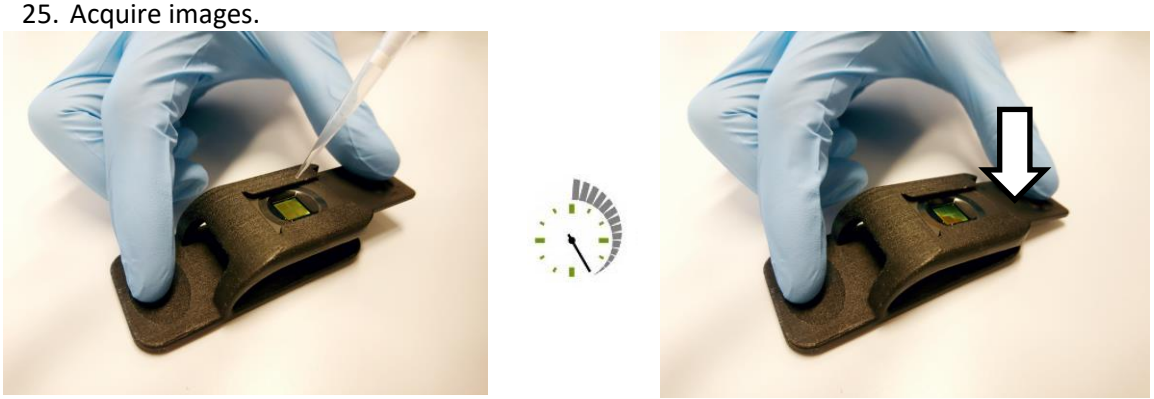

Figure 2: Permeabilization, washing, staining and fixation of the cells are all performed using the sieve staining holder. Solutions can be applied directly on the sieve (left). The solution will stay on the sieve during incubation. By gently pushing down the end of the slide, the sieve will come in contact with the absorber underneath and this will absorb the solution (right).