Filtration and staining protocol

Mice blood samples

Version 1.1

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VyCAP

Reagents

1. Filtered PBS/BSA 1%.
2. Circulating Tumour Cell TransFix/EDTA Vacuum Blood Collection Tubes (Cytomark, CTC-TVT-09)
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 detection of human CTC in mice :
   - Anti Human Pan Cytokeratin CK-11 PE (Cell Signaling Technology, #5075S, clone C-11)
   - Anti Human Pan Cytokeratin AE1/AE3 eFluor 570 (eBioscience, 41-9003-82, clone AE1/AE3)
   - Anti Mouse CD45.2 APC (eBioscience, 17-0454-82, clone 104)

Sample preparation

1. Acquire 0.1 – 1 mL mice blood.
2. Add 5µl of the stabilizer that is inside the Circulating Tumour Cell TransFix/EDTA Vacuum Blood Collection tube, to 100 µl of blood.
3. Leave the sample on the lab bench overnight at RT.

Sample prepartion

1. Dilute the sample 10 fold, with PBS + BSA 1%
2. Pre-filter the diluted sample using CellTrics cell strainers, mesh size 20µm, preferably 20µm (Sysmex, product number 04-0042-2315 (20 µm))
3. Wash the CellTrics cell strainer twice with 2 ml PBS + 1% BSA
4. Continue with the filtration.

Filtration

1. Set the pressure to 25mbar.
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 (prod # FS-510-60) on the pump-unit.
4. Switch ON the pump.
5. Transfer the pre-filtered mouse 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 fluid that is present on the sieve by gently pressing down the slide and sieve on the absorber. There are cases that the absorber is unable to remove the excess blood from the sieve. 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 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 very 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 50 µL PBS+BSA 1% onto the sieve and next push the slide down on the absorber again. This will remove the washing buffer.
5. Release the pressure on the slide (no contact between sieve and absorber anymore)
6. **Fixation** :
   Prepare fixation buffer by adding 100µL of Component A, of the FIX&PERM reagents and add 50µL PBS+BSA 1%. Mix well and add 100 µl of this solution onto the microsieve and incubate 20 minutes at RT.
7. Remove the fixation solution by gently pushing the sieve onto the absorber.
8. Wash the sieve, by adding 50 µL PBS+BSA 1% onto the sieve and next push the slide down on the absorber again. This will remove the washing buffer.
9. **Permeabilization and staining**:
   Prepare a permeabilization/staining cocktail. Take 50µL of component B of the FIX&PERM reagents. Table below displays the volumes to be added to 50µl of component of each of the immunofluorescence labels

Component	Volumes (µL)
Anti Human Pan Cytokeratin CK-11 PE (Cell Signaling Technology, #5075S, clone C-11)	0.5
Anti Human Pan Cytokeratin AE1/AE3 eFluor 570 (eBioscience, 41-9003-82, clone AE1/AE3)	0.5

Anti Mouse CD45.2 APC (eBioscience, 17-0454-82, clone 104)	2.0

Transfer the whole 55µL PERM / Staining cocktail onto the microsieve and incubate for 20 minutes at RT.

10. Remove the staining solution by gently pushing the microsieve down onto the absorber.
11. Apply 50µL PBS/BSA 1% and incubate 5 minutes at RT to remove unbound antibodies.
12. Remove the solution by gently pushing the microsieve down onto the absorber.
13. Remove the slide from the staining holder and insert it again upside down (the backside of the sieve is now facing the user.
14. Add 10µL 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.
15. 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).
16. 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.
17. Place a VyCAP cover glass (prod # CG-10) onto the front side.
18. Incubate 10 minutes
19. Acquire images

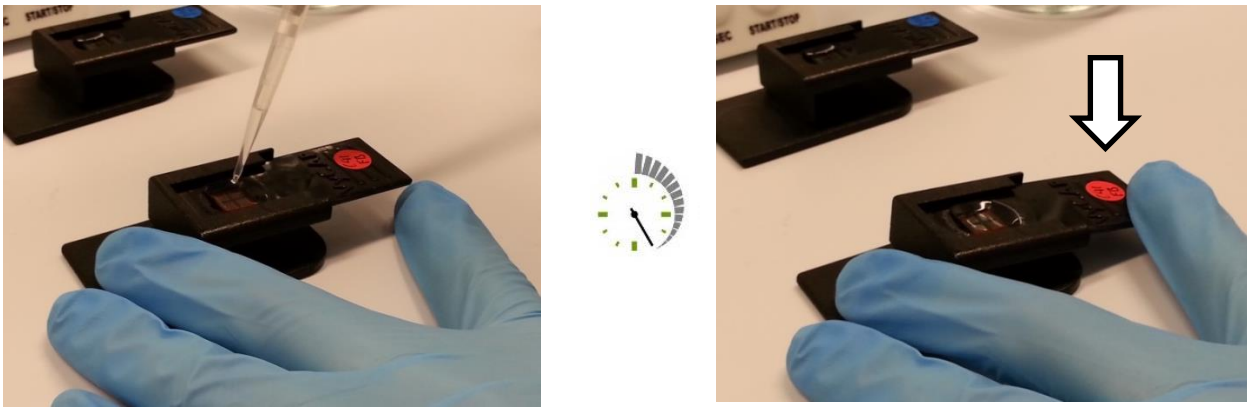

Figure 2: Permeabilization, washing, staining and fixation of the cells are all performed using the sieve staining holder. Solutions can be applied directly on the sieve (left). The solution will stay on the sieve during incubation. By gently pushing down the end of the slide, the sieve will come in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