Experimental Protocol

CTCs enrichment protocol RosetteSep™

Total time for experiment: minutes

Reagents:

RosetteSep™ Enrichment Cocktail, StemCell Technologies, #15127
SepMate™ tubes
PBS + 2% FBS
RNAlater® Solution, Ambion #AM7020
FIX&PERM Cell Fixation and Permeabilization Kit (Nordic MUbio, GAS-002)
Anti-Pan Cytokeratin CK-11 PE (Cell Signalling 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, clone HI30)
Hoechst 33342 100x diluted (Life Technologies, H3570)
50ml centrifuge tubes
10ml serological disposable pipettes
Plastic pasteur pipettes
Sterile pipette tips
1,5 ml eppendorf tubes

Instruments and tools:
Centrifuge, capable of ~1200xg with a swing bucket rotor
p200 and p1000 pipette
Pipetting aid

Prior considerations:
Samples should ideally be processed within 2 hours from the time of extraction.

Steps:

- Add RosetteSep™ Enrichment Cocktail to whole blood: 50 µL/mL of sample (for 10 mL of blood, 500 µL of cocktail).
- Mix (invert the tube gently three times).
- Incubate for 20 minutes at RT.
- Layer SepMate™ tube with 15 mL density medium.
- Dilute sample with an equal volume of PBS + 2% FBS and mix gently (to 20 mL).
- Layer the diluted sample on top of the density medium slowly by the wall´s tube.
- Centrifuge for 20 minutes at 1200 xg at RT, with the brake off.
- Collect the supernatant by turning the tube quickly in a clean falcon (50 mL) and centrifuge 5 minutes, 1200xg with brake. Aspirate supernatant.
- Wash enriched cells with 10 mL of PBS + 2% FBS and centrifuge 5 minutes, 1200xg with brake (2x ideally).
- Aspirate supernatant carefully and resuspend the pellet in 200-400uL of RNAlater or culture medium. For IF aspirate the supernatant of the sample until ~50µl is left.

CTCs detection by Immunofluorescence

- After RosetteSep™ enrichment, the cells will be fixed and stained with FIX&PERM Cell Fixation and Permeabilization Kit (Nordic MUbio, GAS-002).
  For staining the cells, a staining solution containing the following components is recommended:
  -Fix&Perm Component B 100 µL
  -Anti-Pan Cytokeratin CK-11 PE (Cell Signalling Technology, #5075S, clone C-11) 1 µL
  -Anti-Pan Cytokeratin AE1/AE3 eFluor 570 (eBioscience, 41-9003-82, clone AE1/AE3) 1 µL
  -Anti-CD45 APC (eBioscience, 17-0459-41, clone HI30) 3 µL
  -Hoechst 33342 100x diluted (Life Technologies, H3570) 1 µL
  Total volume 106

Steps:

- Add the entire volume of staining solution (106µl) to the washed sample (50µl). Mix by slowly pipetting up and down.

- Incubate the sample at room temperature for 30 minutes.
- Wash by adding 10mL PBS to the sample and spin off at 600xg for 8 minutes.
- Aspirate the supernatant of the sample until 500 µL is left.
- Analyze the sample using a fluorescence microscope.

CTCs selection:

Only round/oval morphology with visible DAPI staining, CK positive (or alternative markers) and CD45 negative can be considered as CTCs.

Contact:

…@ciberonc.es