

Adhesion Assay for Murine Bone Marrow Hematopoietic Stem Cells

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[Abstract] Hematopoietic stem cells (HSCs) are defined by their functional abilities to self-renew and to give rise to all mature blood and immune cell types throughout life. Most HSCs are retained in a non-motile quiescent state within a specialized protective microenvironment in the bone marrow (BM) termed the niche. HSCs are typically distinguished from other adult stem cells by their motility capacity. Movement of HSCs across the physical barrier of the marrow extracellular matrix and blood vessel endothelial cells is facilitated by suppression of adhesion interactions, which are essential to preserve the stem cells retained within their BM niches. Importantly, homing of HSCs to the BM following clinical transplantation is a crucial first step for the repopulation of ablated BM as in the case of curative treatment strategies for hematologic malignancies. The homing process ends with selective access and anchorage of HSCs to their specialized niches within the BM. Adhesion molecules are targets to either enhance homing in cases of stem cell transplantation or reduce BM retention to harvest mobilized HSCs from the blood of matched donors. A major adhesion protein which is functionally expressed on HSCs and is involved in their homing and retention is the integrin $\alpha 4\beta 1$ (Very late antigen-4; VLA4). In this protocol we introduce an adhesion assay optimized for VLA4 expressing murine bone marrow stem cells. This assay quantifies adherent HSCs by flow cytometry with HSC enriching cell surface markers subsequent to the isolation of VLA4 expressing adherent cells.

Keywords: Very late antigen 4, Integrin $\alpha 4\beta 1$, Adhesion-assay, Endothelial protein C receptor (EPCR), Long-term repopulating hematopoietic stem cells (LT-HSC), Flow cytometry

[Background] HSCs are mostly retained in the BM and are regulated by adhesive interactions with their microenvironment, the niche. In this way HSCs are kept in a non-motile quiescent state which protects them from DNA damaging agents (Boulais and Frenette, 2015; Mendelson and Frenette, 2014; Miyamoto *et al.*, 2011; Morrison and Scadden, 2014). The defining properties of HSCs are their functional ability to durably repopulate the irradiated BM of transplanted recipients, which requires their homing, self-renewal and developmental potential (Gur-Cohen *et al.*, 2016). Since adhesion gives rise to activation of intracellular signaling pathways, the type of interaction can mirror the developmental state and behavior of the cells (Sugiyama *et al.*, 2006). Adhesion assays are methods to distinguish between adhesive and non-adhesive cells. In this protocol we introduce a cell adhesion assay under static conditions that separates VLA4 expressing adhesive cells from non-adhesive cells, which are quantified by FACS analysis.

In mouse, hematopoietic stem and progenitor cells (HSPCs) are enriched in a population that lacks lineage markers (Lin; CD8a, CD4, GR1, B220, TER-119, CD11b), and expresses c-Kit (K) and Sca-1

(S). Hence, these cells are also called Lin⁻ Sca-1⁺ c-Kit⁺ (LSK) cells (Adolfsson *et al.*, 2001; Okada *et al.*, 1991; Spangrude *et al.*, 1988). EPCR (endothelial protein C receptor) has been identified as a stem cell marker also in various other tissues (Balazs *et al.*, 2006; Iwasaki *et al.*, 2010; Kent *et al.*, 2009; Ramalho-Santos *et al.*, 2002; Wang and Gerdes, 2015).

Adhesion molecules play a major role in the retention and egress of these HSCs in the BM and to the blood circulation. VLA4 is a receptor for both fibronectin and VCAM-1 and is expressed by most leukocytes, as well as by some non-hematopoietic cells (Hemler *et al.*, 1990), while its expression is higher on murine BM EPCR⁺ LT-HSCs as compared to EPCR negative progenitor cells and circulating LT-HSC (Gur-Cohen *et al.*, 2015). It has long been proposed that VLA4 expression by LT-HSCs might be important for binding and detachment of stem cells within the human BM microenvironment. Inhibition of VLA4 or VCAM-1 binding by neutralizing antibodies causes mobilization of HSPCs from the BM to the blood circulation of mice and primates (Craddock *et al.*, 1997; Papayannopoulou *et al.*, 1995) which is consistent with the notion that VLA4 is crucial for CXCL12/CXCR4-mediated LT-HSC quiescent retention in the BM (Papayannopoulou *et al.*, 1995; Papayannopoulou and Scadden, 2008). In addition to HSPC BM retention, VLA4 is also essential for murine HSPC BM homing (Papayannopoulou and Craddock, 1997). VLA4 possesses different conformations that correlate with its affinity states (Alon *et al.*, 1995; Chen *et al.*, 1999; Feigelson *et al.*, 2001) which are influenced by divalent cations and inside-out signaling (Chigaev *et al.*, 2003; Chigaev *et al.*, 2011). The majority of VLA4 affinity inside-out signaling is mediated by G-protein coupled receptors (Laudanna *et al.*, 2002; Chigaev *et al.*, 2008; Arnaout *et al.*, 2007). Furthermore, elevation of intracellular nitric oxide (NO) was shown to cause cGMP-mediated inhibition of VLA4 affinity (Chigaev *et al.*, 2011). We have previously shown two different pathways, the aPC-EPCR-PAR1 and the thrombin-PAR1 axis, which regulate the NO level up and down, respectively. Thereby, these pathways influence a number of intracellular molecules including Cdc42, CXCR4 and VLA4 leading to retention or mobilization of HSPCs (Gur-Cohen *et al.*, 2015). As described by Gur-Cohen *et al.* (2015), we herein propose the VLA4 mediated adhesion assay for EPCR⁺ stem cells as a powerful tool to predict LT-HSC retention potential to their bone marrow niches.

Materials and Reagents

1. Tissue culture 6-well plates (Corning, Costar®, catalog number: 3516)
2. Nunc™ 8.8 cm² Petri dish (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 153066)
3. 1 ml slip tip Sub-Q syringe with disposable 26 G x 5/8 inch needle (BD, catalog number: 309597)
4. Autoclaved pipet tips:
 - a. 200-1,000 µl blue, gilson tip (Greiner Bio One, catalog number: 740290)
 - b. 200 µl universal tip (Neptune Scientific, catalog number: 2100.N)
 - c. 0.5-10 µl pipet tips (Corning, Axygen®, catalog number: T-300)
5. FACS tubes (Corning, Falcon®, model: 352054)

6. Sterilized 40 µm pore-sized nylon mesh (Sinun Tech, catalog number: Plymer Screends) (see Recipes)
7. Mice
8. Fibronectin (Sigma-Aldrich, catalog number: F0895)
9. Human CXCL12 (human SDF-1 alpha) (reprokine™, catalog number: RKP48061) or (PeproTech, Rocky Hill, NJ, USA)
10. BSA (Sigma-Aldrich, catalog number: A9647)
11. Lymphoprep™ Ficoll (STEMCELL Technologies, catalog number: 07861)
12. Antibodies:
 - a. EPCR PE (Affymetrix, eBioscience, catalog number: 12-2012-82) or EPCR PerCP-eFluor 710 (Affymetrix, eBioscience, catalog number: 46-2012-80) or EPCR Biotin (Affymetrix, eBioscience, catalog number: 13-2012-82) combined with streptavidin-PE (BioLegend, catalog number: 405203)
 - b. Sca-1 PE (BioLegend, catalog number: 108108) or Sca-1 PE/Cy7 (BioLegend, catalog number: 108114)
 - c. c-Kit APC (BioLegend, catalog number: 105812)
 - d. Lineage antibodies

Note: This is a set of antibodies that target the antigens i-vi listed below which are cell lineage markers. In mice these markers do not occur on stem and progenitor cells.

 - i. CD8a FITC (BioLegend, catalog number: 100706)
 - ii. CD4 FITC (BioLegend, catalog number: 100406)
 - iii. GR1 FITC (BioLegend, catalog number: 108406)
 - iv. B220 FITC (BioLegend, catalog number: 103206)
 - v. TER-119 FITC (BioLegend, catalog number: 116206)
 - vi. CD11b FITC (BioLegend, catalog number: 101206)
 - vii. Or Lineage Cocktail-Biotin (Miltenyi Biotec, catalog number: 130-092-613) combined with streptavidin-FITC (BioLegend; catalog number: 405202)

Note: The combination of fluorophores used for the experiment needs to be adjusted according to the experimental demands.
13. 1x Dulbecco's phosphate buffered saline without calcium and magnesium (PBS^{-/-}) (generated from 10x PBS, see Recipes) (Biological Industries, catalog number: 02-023-5A)
14. Roswell park memorial institute (RPMI) 1640 medium ([+] 300 mg/L L-glutamine, [+] 25 mM HEPES) (Thermo Fisher Scientific, Gibco™, catalog number: 52400025)
15. Fetal bovine serum (FBS) (Thermo Fisher Scientific, Gibco™, catalog number: 12657-029)
16. Penicillin-streptomycin (Pen-Strep) solution (Biological Industries, catalog number: 03-031-1B)
17. L-glutamine solution (Biological Industries, catalog number: 03-020-1B)
18. Gentian violet (Sigma-Aldrich, catalog number: G2039)
19. Acetic acid (Sigma-Aldrich, catalog number: ARK2183)
20. 0.1 M sodium azide solution (Sigma-Aldrich, catalog number: 08591)

21. Acetone (Sigma-Aldrich, catalog number: 40289)
Note: This product has been discontinued.
22. Ethanol (Sigma-Aldrich, catalog number: 24103)
Note: This product has been discontinued.
23. Cell dissociation solution (Sigma-Aldrich, catalog number: C5914)
24. ddH₂O
25. Coating solution (see Recipes)
26. Blocking solution with 2% (m/v) BSA (see Recipes)
27. Complete RPMI medium (see Recipes)
28. Turk's solution (see Recipes)
29. FACS buffer (see Recipes)

Equipment

1. Heracell™ 150i CO₂ incubator (Thermo Fisher Scientific, Thermo Scientific™, model: 150i CO₂ incubator)
2. Autoclave
3. Scissors
4. Forceps
5. Refrigerator (4° C)
6. Centrifuge (Eppendorf, model: 5810R)
7. Centrifuge swing-bucket rotor A-462 4 x 250 ml rectangular buckets (Eppendorf, catalog number: 5810709008)
8. Adapters (Eppendorf, catalog number: 5810752000)
9. Finnpiptette model 4500 single channel pipette:
 - a. 0.5-10 µl (Thermo Fisher Scientific, Thermo Scientific, catalog number: FA-10R)
 - b. 5-40 µl (Thermo Fisher Scientific, Thermo Scientific, catalog number: FA-40R)
 - c. 20-200 µl (Thermo Fisher Scientific, Thermo Scientific, catalog number: FA-200R)
 - d. 100-1,000 µl (Thermo Fisher Scientific, Thermo Scientific, catalog number: FA-1000R)
10. Inverted light microscope (Olympus, model: CHK2-F-GS)
Note: This product has been discontinued by the manufacturer.
11. Counting chamber/hemocytometer (Reichert Bright-Line) (Sigma-Aldrich, Bright-Line™, model: Z359629)
12. Flow cytometer (model optional):
 - a. MACSQuant VYB instrument (Miltenyi Biotec, model: 130096116)
 - b. FACSCalibur instrument (BD)
 - c. FACS LSRII instrument (with all four fixed-aligned lasers) (BD)

Software

1. CellQuest software
2. FACSDiva software
3. FlowJo (Tree Star or V10) or MacsQuant

Procedure

A. Coating and preparation

1. Coat tissue culture 6-well plates with 25 µg/ml fibronectin and 2.5 µg/ml CXCL12 overnight at 4 °C. For the coating, it is recommended to prepare a mix of fibronectin and CXCL12 in PBS^{-/-} (coating medium) and to calculate the amount needed so that each well will be covered with 500 µl coating medium. Fibronectin stock (1 mg/ml) and CXCL12 (50 µg/ml) were stored at -20 °C.
2. After overnight incubation, discard the coating medium gently and wash the plates with 1 ml PBS^{-/-} for a few seconds. Repeat washing 3 times.
3. Perform blocking with 2% BSA in PBS^{-/-} for 30 min at room temperature. Blocking solution should cover each well of the plates, and therefore it is recommended to use a minimum of 500 µl blocking solution for each well.
4. Discard the blocking solution gently and wash plates with 1 ml PBS^{-/-} for a few seconds while tilting the plates slowly. Repeat washing 3 times.
5. Plates are ready to be seeded with BM mononuclear cells.

B. Obtaining bone marrow mononuclear cells

1. Sacrifice desired mice by CO₂ euthanasia or cervical dislocation.
2. Extract the femurs, tibias and pelvises using forceps and sharp scissors. Place the bones in a small dish supplemented with PBS^{-/-}.
3. Flush total bone marrow cells from the bone cavity with 1-2 ml PBS^{-/-} (per mouse) using a 1 ml syringe and 16 G needle (Figure 1). Obtain single cell suspension by resuspending the cell solution using the same syringe.

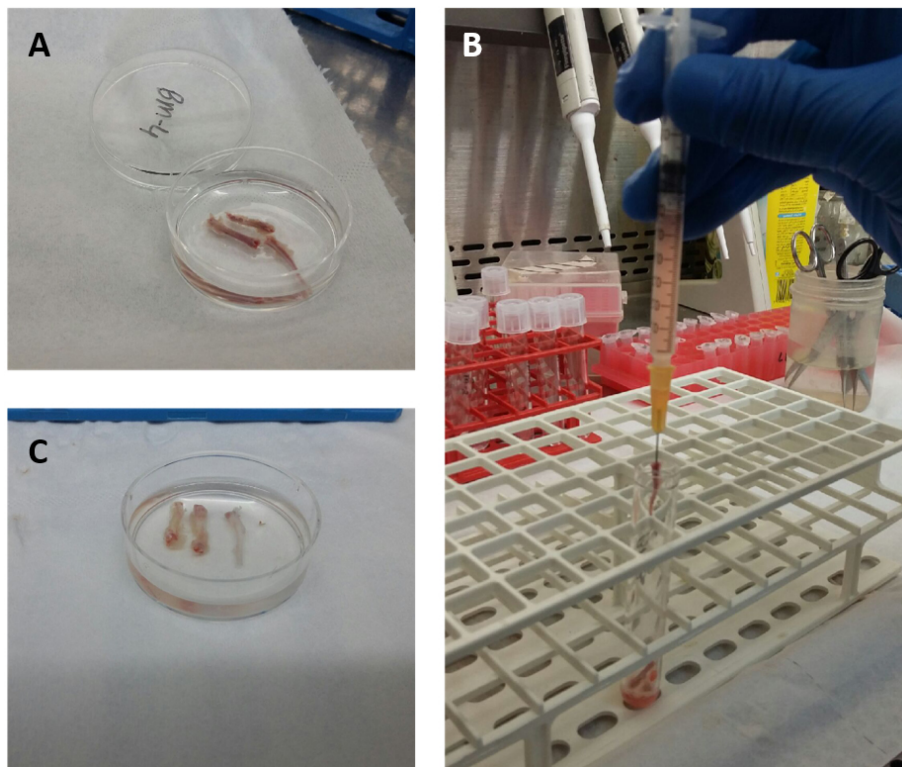


Figure 1. Process of flushing bone marrow out of murine tibia. The red color of the extracted bones indicates the presence of the BM (A). Drilling the needle into the bone and flushing the BM with PBS out of the bone (B) until the bones look white enough (C).

4. Carefully pipet (very slowly) ficoll to the bottom of each tube with a dilution ratio of 1:2 to the bone marrow cells (*i.e.*, if you have a total of 2 ml bone marrow cells in PBS, pipette 1 ml ficoll to the bottom of the tube) (Figures 2A and 2B).

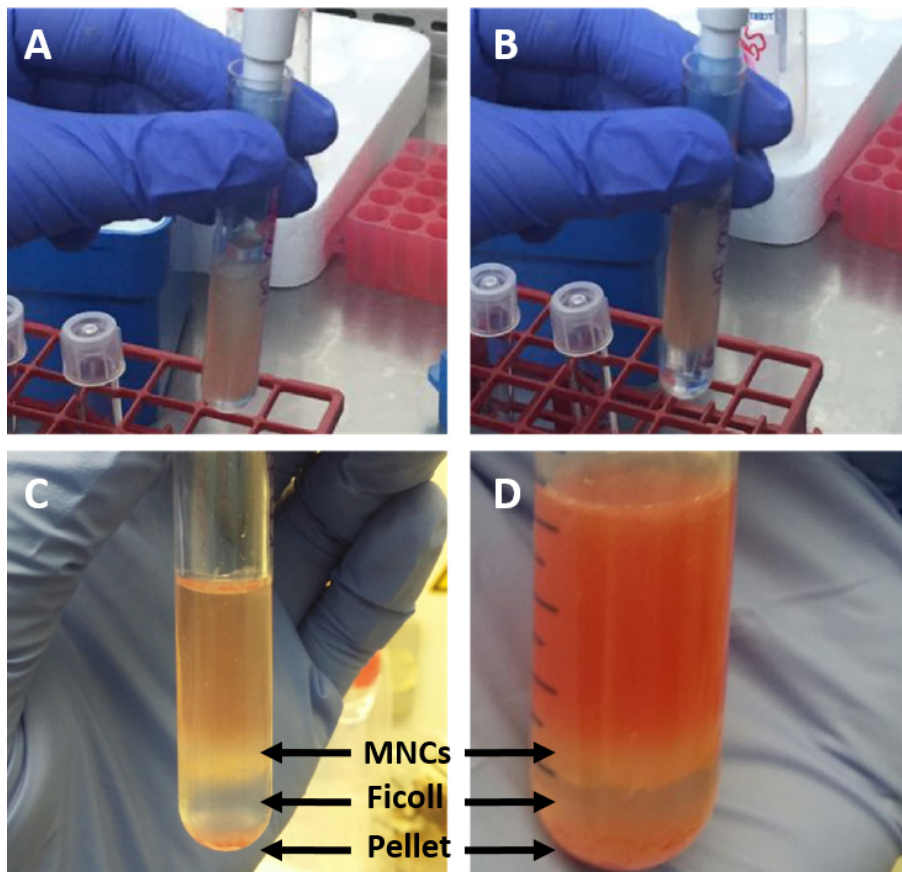


Figure 2. Process of MNC isolation from BM. Ficoll is added to the bottom of the tube underneath the suspended BM (A) forming a lower clear ficoll phase and an upper cell suspension phase (B). After centrifugation the two phases are separated by thin white phase consisting of MNCs (C) which is more prominent the more BM is used for the isolation (D).

5. Centrifuge the cells at $652 \times g$ for 25 min, without a brake (important) at room temperature (mononuclear cells will not be separated if the centrifuge is cold).
6. Carefully remove the tubes from the centrifuge. At the middle phase a fine white ring-like shape should form, containing the bone marrow mononuclear cells (Figures 2C and 2D)
7. Carefully, discard the fluids on top of the ring with a pipet, while avoiding touching the mononuclear fraction. Collect the ring of cells with 200 μ l pipette tip into a new FACS tube.
8. Wash the collected mononuclear cells with 2 ml PBS^{-/-} and centrifuge the cells at $452.8 \times g$ for 5 min.
9. Discard the supernatant and resuspend the cell pellet in 1 ml complete RPMI (see Recipes).
10. Count the cells by diluting the cells 1:10 with Turk's solution. Count the cells under the inverted light microscope using a hemocytometer. From one mouse typically one will get 20-40 million mononuclear cells.

C. Adhesion assay (Figure 3)

1. Seed bone marrow mononuclear cells at a density of 5×10^6 cells per 1 ml complete RPMI medium.
2. Allow the cells to adhere to the coated plates for 2 h at 37 °C in an incubator.
3. Collect non-adherent cells to a new FACS tube by gently rinsing each well with complete RPMI medium. Make sure not to touch the bottom and only add RPMI by placing the pipet tip against the wall of each well.
4. Gently wash the plates 3 times with PBS^{-/-} by tilting the plates back and forth. Make sure not to touch the bottom and only add PBS^{-/-} by placing the pipet tip against the wall of each well.
5. To collect the adherent cells, incubate the plates with cell dissociation buffer for 10 min at 37 °C in the incubator.
6. Monitor the cells under the inverted microscope. If cells are still attached to the bottom, resuspend the cells by using pipet and incubate the plate for additional 5-10 min in the presence of the cell dissociation buffer.
7. Once the cells are detached from the well, vigorously rinse each well using a pipet and collect the adherent fraction to a new FACS tube. Subsequently, add 1 ml ice cold PBS^{-/-} to each well, rinse thoroughly and collect the fluids into the adherent cell fraction to a different FACS tube.
8. Monitor the cells under the inverted microscope. If additional cells remained on the plate, vigorously rinse each well again with ice cold PBS^{-/-} and keep collecting the fluids into the adherent cell fraction.
9. Centrifuge the adherent and nonadherent cell fractions at 452.8 x g, for 5 min.
10. Discard the supernatant carefully.
11. Add 100 µl PBS to each pellet. The cells are now ready to be stained and analyzed.

D. FACS staining and analysis (Figure 4)

1. Add 1 µl of each lineage antibody (CD8a FITC, CD4 FITC, GR1 FITC, B220 FITC, TER-119 FITC, CD11b FITC) and 2 µl Sca1 PeCy7, cKit APC and EPCR PE antibodies to remaining solution on the bottom of each tube.

Note: Do not forget to include a negative control in order to be able to distinguish between the autofluorescence and positive signal during the flow cytometry.

2. Resuspend the pellets by vortexing the tubes briefly.
3. Leave the samples at 4 °C for 30 min.

Note: If biotin-coupled antibodies are used, resuspend the cells in 1 ml PBS after the incubation at 4 °C and repeat the steps from C9 until this step. At step D1 add 1 µl of FITC-coupled streptavidin to the remaining solution of each tube.

4. Resuspend each sample in 1 ml FACS buffer and filter through a 40 µm mesh into a new tube to avoid clots during flow cytometry.
5. Centrifuge the sample at 452.8 x g for 5 min.

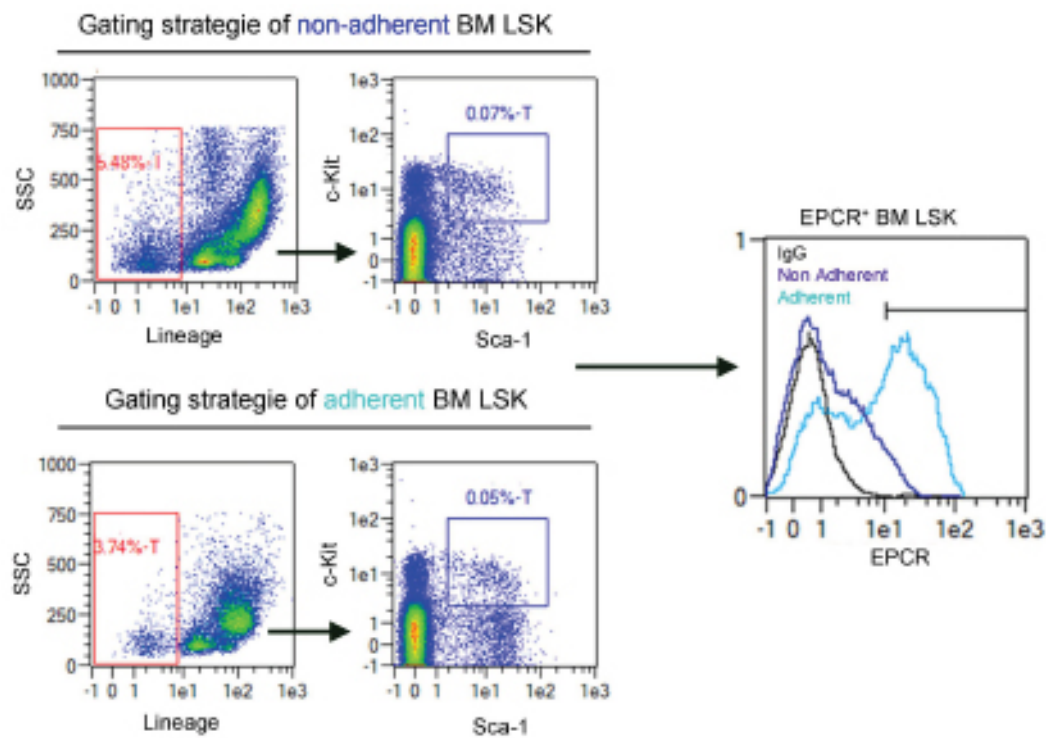


Figure 5. Gating strategy of EPCR positive bone marrow LSK cells (adapted from Supp. Figure 5a of Gur-Cohen *et al.*, 2015)

Data analysis

For further analysis information concerning gating strategies and statistical analysis you can consult the article Gur-Cohen *et al.* (2015) at the following link: <http://www.nature.com/nm/journal/v21/n11/full/nm.3960.html>.

Recipes

- 1x PBS^{-/-}
Add 50 ml of 10x PBS to 450 ml of dH₂O
- Coating solution
1x PBS with 25 µg/ml fibronectin and 2.5 µg/ml CXCL12
- Blocking solution with 2% (m/v) BSA
Add 2 g of BSA per 100 ml 1x PBS
- Complete RPMI medium
88% RPMI 1640 medium
10% FBS
1% Pen-Strep solution
1% L-glutamine solution

5. Turk's solution
50 mg of gentian violet
5 ml of acetic acid
495 ml of ddH₂O
Dilute gentian violet in acetic acid and ddH₂O
6. FACS buffer
Note: Mix this buffer in a plastic bottle.
450 ml ddH₂O
18 µl sodium azide
50 ml 10x PBS
5 ml FBS
7. Sterilized 24 µm pore-sized nylon mesh
 - a. Cut nylon mesh into approx. 3 x 3 pieces
 - b. Incubate the meshes for 20 min at room temperature in acetone under the chemical hood
 - c. Incubate the meshes for 20 min at room temperature in absolute ethanol
 - d. Let the meshes dry overnight
 - e. Sterilize the meshes by autoclaving them at 121 °C for 60 min

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