Bone marrow, spleen and tumor collection for flow cytometry analysis

Javier Rodríguez-Baena

Abstract

Citation: Javier Rodríguez-Baena Bone marrow, spleen and tumor collection for flow cytometry analysis. **protocols.io** dx.doi.org/10.17504/protocols.io.kjecuje
Published: 01 Nov 2017

Materials

DMEM, High Glucose 11965-092 by Life Technologies

Protocol

BONE MARROW

Step 1.
Kill the animal by cervical dislocation and disect into a tissue culture hood. Clean the animal by spraying with 70% EtOH.

BONE MARROW

Step 2.
Take the ventral midline incision to the hips and then divide the cut to both legs until you reach both uncles. Remove as much skin and fur as you can and completely peel the animal from hips down.

BONE MARROW

Step 3.
Cut both legs from the hipbones. If you want to preserve the material, immerse it in a 15ml conical tube with enough PBS to cover it and avoid drying. Legs can be preserved for several hours on ice.

BONE MARROW

Step 4.
In a 100 mm or 150 mm plate place the leg and start removing the flesh covering femur and tibia using forceps, scissors or a scalpel.

BONE MARROW

Step 5.

Twist and distort the femur from the knee and tibia and separate them using forceps. Prevent the bone fracture of damage of the bone marrow will be affected.

BONE MARROW

Step 6.

Using strong scissors, cut the metaphysis and epiphysis (head of the femur from hip and knee respectively) and allow the inside of the shaft to be visible, it is red and rounded.

BONE MARROW

Step 7.

Holding the bone by the middle shaft take 1-2 ml of sterile PBS with a 2 ml syringe and a 23g needle and introduce it in the shaft. Bend the dish to keep all the material in a corner and flush the content down and repeat it 3-4 times until the shaft turns white. The PBS should turn reddish or a red strain should be visible (that is the bone marrow).

BONE MARROW

Step 8.

Disaggregate flushing up and down with the syringe and when the PBS have been homogenised filter to a 50ml conical tube with a 100µm cell strainer.

BONE MARROW

Step 9.

Repeat the procedure with the other leg and add a few ml of fresh PBS to avoid drying.

BONE MARROW

Step 10.

Centrifuge the cells 300g 5 minutes RT.

BONE MARROW

Step 11.

Re-suspend in 10 ml of DMEM and count the cells using a hemocytometer Neubauer chamber. Count $4{\cdot}10^5$ cells per ml and plate, depending on the total media that you will plate in the petri dish or multiwell.

SPLEEN

Step 12.

Kill the animal by cervical dislocation and dissect into a tissue culture hood. Clean the animal by spraying with 70% EtOH.

SPLEEN

Step 13.
Take the ventral midline incision, extract the spleen and remove all the fat tissue around. If part of the spleen is needed for other analysis, cut it with a scalpel and maintain at least a half of the spleen in 1mL of cold collagenase in a 15mL conical tube, until extract the spleen of all animals, for flow cytometry. Spleen can be preserved for several hours on cold collagenase because the enzyme acts at 37ºC.

AMOUNT
1 ml Additional info: Collagenase

NOTES
Javier Rodríguez-Baena 01 Nov 2017

The longer it takes to preserve the samples the worst the outcome will be

SPLEEN
Step 14.
Put a 70µm cell strainer on a bacterial plate and place the spleen on the cell strainer without media or collagenase. Do the mechanical disaggregation crushing it with a plastic plunger of a 3mL syringe. Triturate the spleen until there is just connective tissue on the cell strainer.

SPLEEN
Step 15.
Clean the cell strainer and the bacterial plate with the 1mL cold collagenase where the spleen was maintained to have as many cells as it was possible in the cold collagenase. Put the same cell strainer in a 50mL conical tube, clean it with the cell suspension (in collagenase) that was left in the bacterial plate and after it, clean again with another 1mL of cold collagenase. Maintain on ice until disaggregate all the spleens.

SPLEEN
Step 16.
To do the enzymatic disaggregation, put the cell suspensions into at 37ºC bath for 1 hour, shaking every 10 minutes. Mechanical and enzymatic disaggregation will allow us to obtain single-cell suspension.

SPLEEN
Step 17.
Prepare a new 50mL conical tube with a new 70µm cell strainer.

SPLEEN
Step 18.
Add 3mL of RPMI-10 to the cell suspension. Pass through a 5mL syringe with a 19.5 g needle 3-4 times and pass the cell suspension through the new cell strainer in the 50mL conical tube.

AMOUNT
3 ml Additional info: RPMI

SPLEEN
Step 19.
Centrifuge 5 minutes at 300g.

SPLEEN
Step 20.
Remove the supernatant, resuspend with 5mL of Red Blood Lysis Buffer and incubate 4 minutes at room temperature.

AMOUNT

5 ml Additional info: ACK Buffer (Red blood lysis buffer)

SPLEEN

Step 21.

Centrifuge 10 minutes at 300g.

SPLEEN

Step 22.

Remove the supernatant and resuspend with 5-10 mL of DPBS (depending if the cell suspension comes from a half or a whole spleen and according to the pellet).

AMOUNT

10 ml Additional info: DPBS

SPLEEN

Step 23.

Count the number of cells using trypan blue.

AMOUNT

10 µl Additional info: Trypan blue

TUMOR

Step 24.

Depending on the tumor origin proceed differently and with caution. This protocol has been stablished for B16F1 mouse melanoma and LLC carcinomas.

TUMOR

Step 25.

Remove the tumour without taking any part of skin and/or connective tissue or fat.

TUMOR

Step 26.

Depending on the nature of the tumour it may need a thorough mechanical disassociation, thus, proceed with with caution within a clean surface as in a cell culture plate with small scissors and/or a blade and dissagregate the piece as smooth as you can

GOTO

To do the enzymatic disaggregation, incubate the sample in cold collagenase -> go to step #19

FLOW CYTOMETRY

Step 27.

The following description is valid for the samples aquired previously, Bone marrow, spleen and tumor cell suspension.

NOTES

Javier Rodríguez-Baena 01 Nov 2017

The description of the antibodies can be applied to every reagent that you have in the laboratory but it should be assay-dependen to take that into consideration.

FLOW CYTOMETRY

Step 28.
Incubate 1x10^{-6}cells per FACS tube. For each sample will be needed 2 tubes to do a triple staining: CD3-CD45R-CD11b and CD11b-F4/80-Gr1. For one sample of each group will be also needed 5 tubes to do a simple staining (CD3, CD45R, CD11b, F4/80 and Gr1) and another one to do the negative control (without antibody).

FLOW CYTOMETRY

Step 29.
Centrifuge 5 minutes at 300g.

FLOW CYTOMETRY

Step 30.
Add 7AAD (1:100) and blocking 2.4G2 (1:100) solutions in FACS buffer in a total volume of 50µL and pipette vigorously. Incubate 5 minutes on ice.

FLOW CYTOMETRY

Step 31.
Add the antibodies to the incubated tube (1:50 in a total volume of 50 µL to use them at a final dilution of 1:100 in a total volume of 100µL) and incubate 25 minutes on ice. 50µL of FACS buffer without antibody will be added to the negative control tube.

FLOW CYTOMETRY

Step 32.
Add 1mL of FACS buffer and centrifuge 5 minutes at 300g.

FLOW CYTOMETRY

Step 33.
Resuspend the stained cells (without pippeting) in 500µL of FACS buffer.

FLOW CYTOMETRY

Step 34.
Cell suspension is ready to be analysed by flow cytometry!