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Protocol status: Working
We use this protocol and it's working

Preparation and cryopreservation of human whole blood samples for analysis by flow cytometry (fresh or after cryobanking)

Wiebke Werner[1], Linda Hammerich[1]

[1]Charité - Universitätsmedizin Berlin

Wiebke Werner
Charité - Universitätsmedizin Berlin

ABSTRACT

This protocol focuses on the preparation of **human whole blood samples** for analysis by flow cytometry.
It provides two options for sample preparation: (1) immediate flow cytometry with **fresh** blood samples or (2) **cryopreservation** of the samples at -80°C using the **Stable-Lyse/Store V2** system from SmartTube Inc. for flow cytometry at a later timepoint.

Note: This protocol does not provide guidelines on how to create a multicolor flow cytometry panel!

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GUIDELINES

1. This protocol is only tested on **EDTA anti-coagulated whole blood**.
2. Experiments for **whole blood samples** are performed at **room temperature**. Nevertheless, if you cannot process your sample directly after collection, keep the collection tubes at **4 °C** until processing, especially if you want to store the **plasma** for later analysis (however, extended storage periods, ie. several hours, might influence frequency and phenotype of specific immune cell types, especially myeloid cells).
3. Work with **filter tips**!
4. When working with **fluorochrome-conjugated antibodies**, avoid bright (sun) light and consider using aluminum foil to protect your samples from light while incubating.

Advantages:

1. Analyze all major immune cell subtypes including **granulocytes** with **flow cytometry**.
2. **No pre-selection/exclusion** of certain immune cell subpopulations (e.g. like when isolating PBMC).
3. The procedure itself is **easier and faster**, as it does not require densitiy-gradient centrifugation.
4. **Ready-to-use** quality-controlled cryo-preservation buffers.

Limitations:

1. As mentioned above, this protocol has only been tested on **EDTA-anticoagulated blood**.

Qualifications:

We recommend **basic experience in wet-lab work** (e.g. how to pipette) to handle this protocol.

MATERIALS

INSTRUCTIONS FOR REAGENT AND BUFFER PREPARATION:

Blocking Buffer:

- transfer 1 gram of BSA to a 50 ml falcon
- add 1 ml of each type of seraum (human, mouse, rabbit, rat)
- add PBS ad 50 ml
- mix until dissolved
- aliquot and store at -20°C

FACS Buffer:

- 500 ml DPBS + 2 mM EDTA (2 ml 0.5M EDTA)

Fixation Buffer (2% PFA in PBS):

- dilute 4% PFA in PBS (1:1)

1X Lysis Buffer:

- 9 ml dH_2O + 1 ml BD Pharm Lyse (1:10)

REAGENTS:

Albumin bovine-serum **Merck MilliporeSigma (Sigma-Aldrich) Catalog #A4503**
(BSA)

1X Dulbecco's Phosphate Buffered Saline (DPBS) **Thermo Fisher Scientific Catalog #14190094**

EDTA - Solution pH 8.0 **Panreac AppliChem Catalog #A3145**

BD Pharm Lyse™ **BD Biosciences Catalog #555899**

4% Paraformaldehyde in PBS **Alfa Aesar Catalog #J61899-AK**

Sera from human **Merck MilliporeSigma (Sigma-Aldrich) Catalog #S2257-1ml**

Sera from mouse **Merck MilliporeSigma (Sigma-Aldrich) Catalog #S3509-1ml**

Normal rabbit serum (invitrogen) **Thermo Fisher Scientific Catalog #10510**

Normal rabbit serum (invitrogen) **Thermo Fisher Scientific Catalog #10710C**

+ **fluorochrome-conjugated antibodies of your choice**
+ **fixable viability stain** (if desired)

REAGENTS ONLY NEEDED FOR CRYOFIXATION:

Stable-Lyse V2 **SMART TUBE Inc. Catalog #Stable-Lyse V2**

Stable-Store V2 **SMART TUBE Inc. Catalog #Stable-Store V2**

DISPOSABLES OF YOUR CHOICE:

- EDTA blood collection tube
- Cryotubes (1,8 ml)
- Microcentrifuge tubes (1,5 ml)

- Pipet filter tips (10, 200 and 1000 µl)
- 5 ml FACS tubes
- caps for FACS tubes

Equipment:

- biosafety cabinet (BSL2)
- centrifuge
- pipets (0,1-2,5 µl, 0.5-10 µl, 10-100 µl, 100-1000 µl)
- pipette controller

SAFETY WARNINGS

If you work with **untested human samples** (e.g. status of Hepatitis B/C and HIV is unknown), all experiments must be carried out in a **Class II biosafety cabinet (with laminar air flow)**. Liquid waste has to be collected and **autoclaved** before disposal, contaminated materials have to be collected and **disposed of separately** from other lab waste as they are potentially infectious.

Use **caps/lids** to close your **FACS tube** when removing them from the biosafety cabinet for centrifugation. Use pipet tips with **filters**.

Depending on your personal safety preferences as well as the specific regulations of your research department/institute/country, there might be other or additional regulations to consider. Contact your **biosafety officer**!

ETHICS STATEMENT

Before you work with human samples, you have to **acquire an human ethics approval** from the local ethics committee of your institution! Every subject/patient has to give **informed consent**!

BEFORE START INSTRUCTIONS

1. Make sure you read the safety warnings regarding **untested human samples**!
2. Make sure an **ethics approval** according to your institution´s guidelines has been obtained before working with subjects or patient samples!
3. Revisit the **materials list** to make sure all the equipment, materials and reagents are available to you.

1 Depending on whether you want to perform flow cytometry on fresh or cryo-preserved samples, you can select different protocol options at this step.
Step 1 includes a Step case.
WB - fresh
WB - stored

Preparations

30m

step case

5m

WB - fresh

Use this protocol if you want to perform flow cytometry immediately after blood sample collection ("fresh").

2 Label your **5 ml FACS tubes**. You might need several tubes per sample, depending on the number of panels and controls you are planning to run.

Note

GOOD TO KNOW - FMO (fluorescence minus one) controls are helpful to discriminate positive and negative (unspecific background fluorescence) signals, especially if the positive cell populations are not distinctly separated from the negative population. In an FMO tube, all fluorochromes in the panel are present except the fluorochrome in question.

3 Prepare the **antibody mix**: For each FACS tube, use 20 µL **blocking buffer** and the appropriate amount of each of your **antibodies**. Always prepare the master mix for one extra sample or add 10% for pipetting errors. 25m

Note

EXAMPLE 1

- 2 blood samples (one full stain and one control each) + 1 extra for pipetting error = 5x mix
- 20 antibodies in panel (1µl needed per sample)
- Blocking Buffer: 5 x 20 µl = 100 µl
- 5 µl per antibody
- final volume of antibody master mix: 200 µl
- mix to add per FACS tube = 20 µl blocking buffer + 20 µl antibodies = 40 µl

EXAMPLE 2

- 2 blood samples (one full stain and one control each) = 4x mix +10%
- 20 antibodies in panel (1µl needed per sample)
- Blocking Buffer: 4x 20 µl = 80 µl (+ 10%) = 88 µl
- Each antibody: 4 µl per antibody +10% = 4,4 µl per antibody
- final volume of antibody master mix: 176 µl
- mix to add per FACS tube = 20 µl blocking buffer + 20 µl antibodies = 40 µl

4 Collect **peripheral blood** by venipuncture directly into a collection tube with **EDTA** and invert several times to mix and ensure proper anti-coagulation

Note

Blood samples should be processed as soon as possible. If immediate processing is not possible, keep samples at 4°C.

Staining (BSL2)

25m

5 **Invert** the EDTA blood collection tube several times to homogenize, especially if the samples have been kept in the fridge. 1m

6 Transfer 200 µL of **whole blood** to each of your prelabeled 5 ml FACS tubes. 30s

7 Add the appropriate concentration of **fixable viability stain** directly to the **whole blood** in each of the FACS tubes and mix well by flicking the tube or vortexing. 1m

Note

HOW TO "FLICK"- Mix well by holding the upper part of the tube between thumb and index finger of one hand and at the same time, gently flick the bottom of the tube repeatedly with the index finger of the other hand.

8 Incubate for 00:05:00 at Room temperature . 5m

9 Add the appropriate amount of the **prepared antibody mix** (see Step 3) to each tube. Gently pipette up and down to mix. 1m

Note

EXPERT TIP - In case you are using FMO control(s), you can prepare the antibody mix for all your tubes without the FMO antibody(ies) and add them later to your full stain tube.

10 Incubate 00:20:00 at Room temperature . 20m

Red blood cell lysis (BSL2) 17m

11 Add 2 mL **1X Lysis Buffer** to each FACS tube and mix well by pipetting up and down. 1m

Note

HOW TO - Works best using a 1000 µl pipet

12 Incubate for 00:10:00 at Room temperature . 10m

13 Add 2 mL **FACS buffer** and mix well by pipetting up and down. 1m

14 Spin 500 x g, Room temperature, 00:05:00 5m

15 **Discard the supernatant by decanting** and resuspend the cell pellet in the remaining buffer by flicking the tube. 2m

Note

HOW TO DECANT - Empty the supernatant with momentum and - while keeping the FACS tube in an upside-down position to not disrupt the pellet on the bottom - dip the tube onto a paper towel to get rid of the excess liquid. Turn the FACS tube upright and gently flick to resuspend.

16 Add 1 mL **1X Lysis Buffer** and mix well by pipetting up and down. 1m

17 Incubate 00:05:00 at Room temperature . 5m

18 Add 2 mL **FACS buffer** and mix well by pipetting up and down. 1m

19 Spin 500 x g, Room temperature, 00:05:00 5m

20 **Discard the supernatant** and resuspend the cell pellet in the remaining buffer by flicking the tube. 2m

Fixation 15m

21 Add 1 mL **Fixation Buffer** and mix well by pipetting up and down. 1m

22 Incubate for 00:10:00 at Room temperature. 10m

23 Add 2 mL **FACS buffer** and mix well by pipetting up and down. 1m

Note

After fixation, samples can be further processed on the bench, a biosafety cabinet is no longer necessary.

24 Spin 500 x g, Room temperature, 00:05:00 5m

25 **Discard the supernatant** and resuspend the cell pellet in the remaining buffer by flicking the tube. 2m

26 Add 200 µL **FACS buffer** and proceed to sample aquisition. 1m

Note

Fixed samples can be stored at 4 °C overnight and recorded the next day. However, keep in mind that fixatives such as formalin can change the autofluorescence of your cells and destabilize fluorochromes, especially tandem dyes. If possible, record you samples directly after fixation.