Nov 18, 2025

Isolation, Cell Counting, and Freezing of PBMCs @ ZeBanC

DOI
https://dx.doi.org/10.17504/protocols.io.kxygx486kl8j/v1

Merz, MP[1], Stege, A[2], ZeBanC - Zentrale Biobank Charité[2]

[1]Berlin Institute of Health (BIH) @ Charité; [2]Charité Universitätsmedizin

Merz, MP: orcid.org/0000-0003-2085-1980;
ZeBanC - Zentrale Biobank Charité: RRID:SCR_023495

ZeBanC - Zentrale Biobank Charité

Charité Universitätsmedizin, Berlin Institute of Health, Ger...

Create & collaborate more with a free account

Edit and publish protocols, collaborate in communities, share insights through comments, and track progress with run records.

Create free account

OPEN ACCESS

DOI: https://dx.doi.org/10.17504/protocols.io.kxygx486kl8j/v1

Protocol Citation: Merz, MP, Stege, A, ZeBanC - Zentrale Biobank Charité 2025. Isolation, Cell Counting, and Freezing of PBMCs @ ZeBanC . **protocols.io** https://dx.doi.org/10.17504/protocols.io.kxygx486kl8j/v1

License: This is an open access protocol distributed under the terms of the **Creative Commons Attribution License**, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

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

Created: November 04, 2025

Last Modified: November 18, 2025

Protocol Integer ID: 231480

Keywords: PBMCs, ZeBanC, EDTA, Heparin, Citrate, PBMC Isolation, LeucoSep, freezing of pbmc, central biobank charité, pbmc, peripheral blood mononuclear cell, zebanc, determination of concentration, cell

Disclaimer

DISCLAIMER – FOR INFORMATIONAL PURPOSES ONLY; USE AT YOUR OWN RISK

The protocol content here is for informational purposes only and does not constitute legal, medical, clinical, or safety advice, or otherwise; content added to **protocols.io** is not peer reviewed and may not have undergone a formal approval of any kind. Information presented in this protocol should not substitute for independent professional judgment, advice, diagnosis, or treatment. Any action you take or refrain from taking using or relying upon the information presented here is strictly at your own risk. You agree that neither the Company nor any of the authors, contributors, administrators, or anyone else associated with **protocols.io**, can be held responsible for your use of the information contained in or linked to this protocol or any of our Sites/Apps and Services.

Abstract

This procedure describes the isolation, determination of concentration, and freezing of PBMCs (Peripheral Blood Mononuclear Cells) from blood (EDTA, heparin, citrate) at the Central Biobank Charité (ZeBanC), unless otherwise specified.

Attachments

F071043_Leucosep_Man... 729KB

cell-counting-accura... 320KB

MAN0019566_Countess_... 7.4MB

Guidelines

The Central Biobank Charite (ZeBanC) works under the best practice laboratory guidelines and ISO:9001 quality standards.
This protocol in its published form has only been validated on machines and chemicals as described here.
In case of doubt, please feel free to consult attached manufacturers SOPs or reach out to us.

Materials

- Ficoll Paque Plus, 500mL (Cytiva #17-1440-03)
- FCS (Fetal bovine/calf serum) (Sigma-Aldrich #F7524-500ml)
- RPMI (Thermo #31870025)
- Dimethylsulfoxide (DMSO) Cell Culture Grade, 250mL (Sigma-Aldrich #D8418-250ml)
- phosphate buffered saline (PBS) without Ca/Mg, 500mL (GIBCO #605294)
- 0.4% trypan blue solution (Fisher Scientific #15250061)
- Isopropanol (to fill Mr. Frosty)

- LeucoSep tubes (Greiner #227290)
- Polypropylen Tubes ("Falcon Tubes"), 50mL (Falcon #352070)
- 10ml serolog. Pipettes (Sarstedt #86.1254.001)
- 25ml serolog. Pipettes (Sarstedt #86.1685.001)
- cell counting slides (for Countess) (Thermo #C10283)
- Mr. Frosty (Thermo #5100-0001)

Equipment

Countess 3 FL Automated Cell Counter	NAME
Automated Cell Counter	TYPE
Thermofisher scientific	BRAND
AMQAF2000	SKU
https://www.thermofisher.com/th/en/home/life-science/cell-analysis/cell-analysis-instruments/automated-cell-counters/models/countess-3-fl.html	LINK

Equipment

Megafuge ST1 Plus MD	NAME
centrifuge	TYPE
Megafuge ST1 Plus Centrifuge	BRAND
https://www.thermofisher.com/order/catalog/product/75009770?SID=srch-srp-75009770	LINK

Equipment

Safe 2020	NAME
bio safety cabinet	TYPE
Herasafe	BRAND
https://www.thermofisher.com/de/de/home/life-science/lab-equipment/biological-safety-cabinets/models.html	LINK
class II A2	SPECIFICATIONS

Safety warnings

Cell separation medium (Ficoll) and trypan blue solution are cytotoxic.

Be aware that this SOP has NOT been tested for BD Vacutainer® CPT™ Mononuclear Cell Preparation Tubes.

Ethics statement

The Central Biobank Charité (ZeBanC) acts as a core facility/service provider for researchers. When appropriate, the ZeBanC requests to see ethical approval and informed consent statements before biospecimen processing.

Before start

Time to isolation:
Ideally, PBMCs are isolated and frozen within 4 hours of blood collection. However, it is possible to process PBMCs up to 24 hours after collection (during this time some cells will be lost to apoptosis).
In this case, whole blood should be stored in the original tube (heparin, EDTA or citrate) at room temperature (approx. 18-22°C) until isolation.

Things to prepare before starting

1h

1 **Prepare LeuoSep Tubes** 5m

- Fill LeucoSep tubes (incl. divider disc) with 15 mL of Ficoll (each) under the bench and seal them (with lid).
- Centrifuge at 1000 x g for 30 seconds (at room temperature) so that all the Ficoll locates below the divider disc.
- If any Ficoll remains above the divider disc, carefully remove it (under the bench) with a pipette and discard it.
- Store filled LeucoSep tubes (closed) at room temperature in a dark place and use within 4–6 weeks.

1.1 **Heat-incativation [HI] of FCS** 45m

- Inactivate FCS in a **water bath at 56°C for 30 minutes**, stirring occasionally.
- Then portion and **freeze (-20°C)**. Label the bottle/tube/aliq. with [HI] and the freezing date.
- After thawing, store at 4°C and use within 4 weeks.

1.2 **Prepare and aliquot freezing medium 1 and 2** 10m

Freezing medium 1 (**consisting of 40% FCS [heat-inactivated] + 60% RPMI**)
o For 15 mL stocks= 5 mL FCS + 7.5 mL RPMI
o For 50 mL stocks= 18 mL FCS + 27 mL RPMI
o Aliquots can be stored at -20°C
o Once thawed, store at 4°C and use within one week

Freezing medium 2 (**20% DMSO + 80% FCS [heat-inactivated]**)
o For 15 mL stocks= 10 mL FCS + 2.5 mL DMSO
o For 50 mL stocks= 36 mL FCS + 9 mL DMSO
o Aliquots can be stored at -20°C
o Once thawed, store at 4°C and use within one week

Isolation of PBMCs (via LeucoSept)

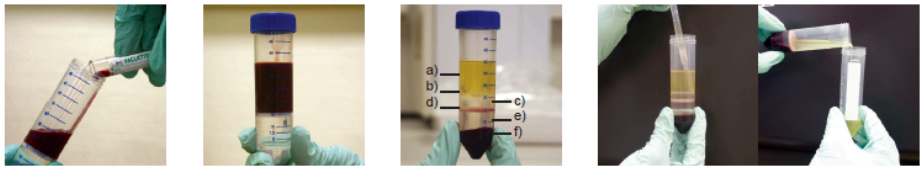
1h 50m

2 Fill up to 20 mL of whole blood (e.g. from two heparin tubes) into a 50 mL **LeucoSep tube**
(equipped with a divider and 15 mL of Ficoll). 20m

Rinse the emptied blood tubes with 5-10 mL of **PBS** and add this to the LeucoSep tube. Finally, fill the LeucoSep tube with PBS to approximately 40-45 mL.

All above procedures should be performed at **room temperature**.
Work quickly, as the separation medium (Ficoll) is cytotoxic.

Procedure



1) Filling with sample material
2) Before centrifugation
3) After centrifugation
4) Harvest by means of a Pasteur pipette or by decanting into another centrifugation tube

1. Pour the anticoagulated sample material (blood or bone marrow aspirate, diluted with balanced salt solution if necessary) directly from the blood sampling tube carefully into the Leucosep tube: 3–8 ml of sample material when using tubes order no.163288, 163289 or 163290; 15–30 ml of sample material when using tubes order no. 227288, 227289 or 227290.
2. Centrifugate 10 minutes at 1000 x g and RT or 15 minutes at 800 x g and RT in a swinging bucket rotor. Switch off brakes of the centrifuge.
3. After centrifugation the sequence of layers occurs as follows (seen from top to bottom): a) Plasma – b) enriched cell fraction (interphase consisting of lymphocytes/ PBMCs) – c) separation medium – d) porous barrier – e) separation medium – f) pellet (erythrocytes and granulocytes). Collection and discarding of the plasma layer fraction up to a minimum remnant of 5 to 10 mm above the interphase helps to prevent contamination of the enriched cells with platelets.
4. Harvest the enriched cell fraction (lymphocytes / PBMCs) by means of a Pasteur pipette or by pouring the supernatant above the porous barrier from the Leucosep tube into another centrifugation tube. The porous barrier effectively avoids recontamination with pelleted erythrocytes and granulocytes.

Pictures and description from Greiner Bio-One Instruction manual LeucoSep

2.1 The layers are separated by **centrifugation at 1000 x g, 10 min, 20°C, without brake**

45m

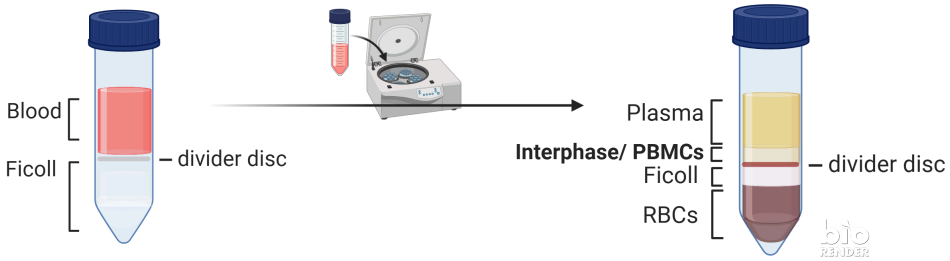
Note

It takes about 35-40 min for the centrifuge to swing out.

This is a good time to prepare a cryo container (Mr. Frosty), the cell counting slides and maybe label your final cryo tubes.

2.2 After centrifugation, the mononuclear cells (primarily lymphocytes and monocytes) are located in the interphase, which is recognizable as a whitish ring. Discard the plasma fraction above (stopp approx. 20mm above the whitish ring to **not disturb the interphase**).
Then, **transfer the interphase** from the LeucoSep tube into a **fresh 50 mL Falcon** tube by pouring. The divider disc (in the LeucoSep tube) prevents contamination with the depleted erythrocytes, granulocytes and Ficoll.

15m



LeucoSep layers before and after centrifugation. Figure © ZeBanC; created with BioRender

Note

When transferring to a new Falcon, it is possible to combine two LeucoSep tubes (same blood draw; do **NOT mix patients/timepoints**).

2.3 To remove Ficoll residues, the collected cells are washed with PBS by centrifugation. For this purpose, the 50 mL Falcon tube (containing the interphase) is filled with **20-30 mL of PBS (@RT)** (total volume approximately 40-45 mL), mixed by inverting several times, and then **centrifuged at 400 x g for 10 min, medium break (@RT).**

20m

2.4 After centrifugation, a **cell pellet should be visible** at the bottom wall of the Falcon tube. **Discard the PBS** (pour off/remove by pipetting) without disturbing the cell pellet.

5m

Note

After this step you can pre-cool the centrifuge to 4°C

2.5 Resuspend the pellet in **10 mL of PBS** (@4°C).
Mix the cells thoroughly with the PBS and immediately **remove 10 µL for cell counting**.

5m

Note

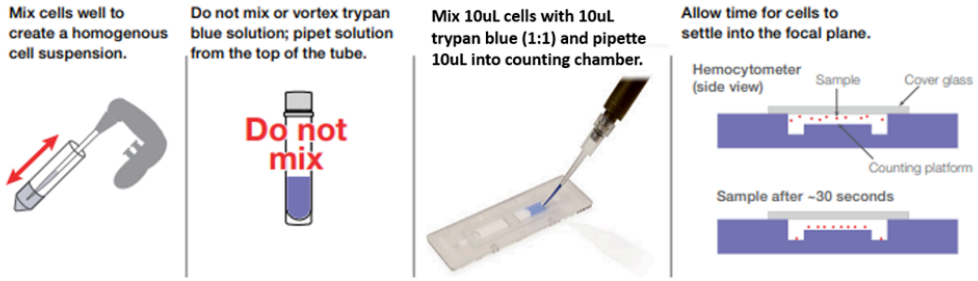
While counting cells, you can already centrifuge the Faclon tube/s. See step 4

Automated cell counting (via Countess3)

7m

3 For cell counting, 10 µl of the cell suspension is taken and mixed with 10 µl of trypan blue solution [ratio 1:1] (in a separate container/small tube).
Dead cells will be stained blue by trypan blue. 10 µl of the trypan blue-stained suspension is pipetted into an opening of the counting chamber and, after approximately 30 seconds, transferred to the automatic cell counter.

5m



Steps for cell counting on the Countess3. © invitrogen/ThermoFisher

3.1 The counting chamber is inserted into the slot of the device (Countess3 FL) with the loaded side (A or B) facing forward. The Countess 3 cell counter focuses automatically (*our settings exclude everything under 8µM as cell depris or RBC*). Counting begins after the Count button is pressed. The relevant result, including the number of viable cells, is displayed as Live (% and concentration per mL).

1m

Inserting counting chambers into the Countess3 FL and starting the counting process. Pictures © ZeBanC

PS: The 1:1 dilution (step 3.1) with trypan blue is automatically factored in by the cell counter

Note

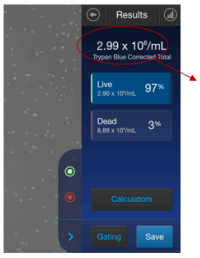
Approximately (0.5 – 3) x 10^6 PBMCs can be expected per mL of human whole blood. For example, using two full heparin tubes (2 x 9 mL), we expect a total cell count of (2-4) x 10^7 cells [= 20–40 x 10^6]. Since we diluted to 10 mL of PBS (step 2.6), the cell counter should show between (2–4) x 10^6/mL. Ideally, over 90% of cells should be viable.

3.2 Calculate the required volume of *complete* freezing medium to adjust to desired cell concentration. 1m

Complete freezing medium consists of **50% freezing medium 1** and **50% freezing medium 2,** which are added in a **two-step procedure** (see steps 4.1 and 4.2

Note

Example: A concentration of 2.99 x 10^6/mL is displayed and we previously dissolved the cell pellet in 10mL PBS (step 2.5), we have 10mL x 2.99x 10^6/mL = **2.99x 10^7** (i.e. 29.9 x 10^6) cells.
If we need a normalized concentration of 1x 10^6/mL (i.e. a million cells per mL), we would have to dilute the cell pellet (after the next centrifugation) with **29mL of complete freezing medium** (=14.5mL freezing medium 1 **+** 14.5mL freezing medium 2).



$$\underset{\text{Conc. c/mL}}{\text{conc}} \times \underset{\text{Volume}}{V} = \underset{\text{Total cell count}}{N}$$

$$\underset{\text{Conc. c/mL}}{2.99 \times 10^6 \frac{1}{mL}} \times \underset{\text{Dilution/Volume}}{10mL} = \underset{\text{Total cell count}}{2.99 \times 10^7}$$

$$\underset{\text{Volume}}{V} = \frac{\underset{\text{Total cell count}}{N}}{\underset{\text{Normalized conc.}}{C}} = \frac{2.99 \times 10^7}{10^6 \frac{1}{mL}} = \underset{\text{Volume}}{29mL} = \begin{matrix}\text{14.5mL freezing medium 1} \\ \text{+ 14.5mL freezing medium 2}\end{matrix}$$

Pictures + Figures © ZeBanC

Freezing of PBMCs (using Mr. Frosty) 47m

4 Centrifuge the 50mL Falcon tube containing the cell suspension (in PBS, 10mL; step 2.5) at **400g, 10min, 4°C,** with medium brake. 10m

Note

Can be started during the cell counting.

4.1 Discard the supernatant (PBS) & pipette off the return flow without disturbing the pellet. Quickly resuspend the pellet with (50% total volume) of **freezing medium 1** (consisting of 40% FCS + 60% RPMI, @4°C). 3m

The pellet can initially be dissolved in a smaller volume (with a smaller pippette); however, the total volume remains the same.

4.2 Then, dropwise, add (50% total volume) of **freezing medium 2** (consisting of 80% FCS + 20% DMSO, @4°C) and mix within the Falcon tube by (slight) swirling motion. If necessary, allow it to run along the inside wall of the Falcon. 3m

Note

Since **freezing medium 2** contains **DMSO**, you will have to **work quick** as soon as its added to the cells.

4.3 Finally, transfer 1.9mL cell solution (each) into 2mL cryotubes. This leaves some room for expansion of fluids while freezing. 1m

Note

At ZeBanC we like to use 2D-barcoded tubes like LVL, FluidX or similar.

4.4 Take a cryo container (Mr. Frosty), transfer the filled cryotubes into the Mr. Frosty (@4°C) and then place the Mr. Frosty in a -80°C freezer. Note the time and date of freezing onto the lid. 10m

The cells should be transferred to a liquid nitrogen tank (-160 to -196°C) within one week, but earliest after 24 hours of storage at -80°C.

Note

Prepare/check the isopropanol level in Mr. Frosty beforehand.
Some cryo containers do not need isopropanol.

4.5 Finally, the data (tube ID, date of preparation, concentration...etc) is entered into the Biobank LIMS system 20m

Protocol references

Isolation:

https://www.gbo.com/fileadmin/imported_from_old/Leucosep_Protocol.pdf (see: Attachments); adapted

https://www.mcgill.ca/neuro/files/neuro/cbig-02-002_v1.0_pbmc_isolation_from_whole_blood_leucosep_method.pdf; adapted

Time to processing:

Lehle S, Völkl S, Seitz K, Goossens C, Emons J, Ruebner M, Uhrig S, Ziegler P, Theuser AK, Beckmann MW, Fasching PA, Huebner H. Effect of delayed isolation of peripheral blood mononuclear cells on cell viability and functionality. BMC Immunol. 2025 Mar 15;26(1):21. doi: 10.1186/s12865-025-00701-y. PMID: 40089714; PMCID: PMC11909936.

Rivas I, Li Z, Irvin J, Huda N, Arzi B, Vapniarsky N. Delayed isolation and cryopreservation have significant effects on the yield and proliferation of feline peripheral blood mononuclear cells. Am J Vet Res. 2025 Jul 14;86(10):ajvr.25.04.0124. doi: 10.2460/ajvr.25.04.0124. PMID: 40675187.

Diane Longo, Brent Louie, Erik Evensen, Rachael E. Hawtin, Alessandra Cesano; Impact of Time From Blood Draw to Peripheral Blood Mononuclear Cell (PBMC) Processing and Cryopreservation on Functional Pathway Activity As Measured by Single Cell Network Profiling (SCNP) Assays. *Blood* 2011; 118 (21): 4922. doi: https://doi.org/10.1182/blood.V118.21.4922.4922

Browne DJ, Miller CM, Doolan DL. Technical pitfalls when collecting, cryopreserving, thawing, and stimulating human T-cells. Front Immunol. 2024 May 15;15:1382192. doi: 10.3389/fimmu.2024.1382192. PMID: 38812513; PMCID: PMC11133553.

Amelia Jerram, Thomas V. Guy, Lucinda Beutler, Bavani Gunasegaran, Ronald Sluyter, Barbara Fazekas de St Groth, Helen M. McGuire; Effects of storage time and temperature on highly multiparametric flow analysis of peripheral blood samples; implications for clinical trial samples. *Biosci Rep* 26 February 2021; 41 (2): BSR20203827. doi: https://doi.org/10.1042/BSR20203827

Freezing media:

Juhl M, Christensen JP, Pedersen AE, Kastrup J, Ekblond A. Cryopreservation of peripheral blood mononuclear cells for use in proliferation assays: First step towards potency assays. J Immunol Methods. 2021 Jan;488:112897. doi: 10.1016/j.jim.2020.112897. Epub 2020 Oct 10. PMID: 33049298.

Storage Temp:

Yang J, Diaz N, Adelsberger J, Zhou X, Stevens R, Rupert A, Metcalf JA, Baseler M, Barbon C, Imamichi T, Lempicki R, Cosentino LM. The effects of storage temperature on PBMC gene expression. BMC Immunol. 2016 Mar 15;17:6. doi: 10.1186/s12865-016-0144-1. PMID: 26979060; PMCID: PMC4791795.

Central Biobank Charité would like to acknowledge the BIH (Berlin Institute of Health) CRU/BeLOVE Unit for sharing their SOP.

Acknowledgements

Central Biobank Charité would like to acknowledge the BIH (Berlin Institute of Health) CRU/BeLOVE Unit for sharing their SOP.