- Standard Operating Procedure 0016-

Preparation and Biobanking of PBMCs using Vacutainer® CPT™ tubes for ChipCytometry

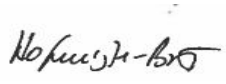
Short Description

This SOP describes how to isolate PBMCs from whole blood for biobanking and later analysis by ChipCytometry.

Versioning

Version Number	1.3	valid from: 2018-11-21
Replaces Version	1.2	valid from: 2018-04-09

Signatures

Author Nancy Stanslowsky	Date 2018-11-20	Signature 
Reviewer Anke Hofmeister-Brix	Date 2018-11-21	Signature 
Approval Christian Hennig	Date 2018-11-21	Signature 

Target Group

	laboratory personnel of Zellkraftwerk; customers of Zellkraftwerk
Internal use only	NO

Table of Content

A. Scope

The scope of this standard operating procedure is to describe how PBMCs are separated from anticoagulated blood using 8 ml Vacutainer® CPT™ tubes, and how the cells are loaded and fixed on ZellSafe™ chips for subsequent testing.

B. Definitions and Abbreviations

- PBMCs: peripheral blood mononuclear cells
- RT: room temperature

C. Personnel Qualifications

Basic education and training

- hygiene requirements;
- wearing and use of protective equipment and clothing;
- handling of potentially infectious materials;
- laboratory design, including airflow conditions;
- prevention of incidents and steps to be taken by workers in the case of incidents (biohazard incidents, chemical, electrical and fire hazards);
- good laboratory practice;
- organization of workflow;
- waste handling;
- use of equipment (operation, identification of malfunctions, maintenance).

The training shall be:

- given before a staff member takes his/her post;
- strictly supervised;
- adapted to take into account any new or changed conditions; and
- repeated periodically, preferably every year.

D. Equipment and Procedure

Principle

PBMCs are separated from whole blood in 8 ml Vacutainer® CPT™ tubes, resuspended in wash buffer, and then pipetted into ZellSafe™ chips, using a standard procedure demonstrated in the video available at this website: https://www.youtube.com/watch?v=X1bXN2rbyTU&t. **In case of deviation between this SOP and the video, you must strictly adhere to the steps described in the present document. Avoid pipetting air through the chip channel as soon as a sample is loaded.**

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Equipment and Materials

- Pipettes and pipette tips (non sterile)
- Centrifuge with swinging bucket rotor and tube adapters for 16x125 mm tubes
- BD Falcon® round-bottom tubes with a cap (BD Falcon, Cat.# 352058)
- ZELLKRAFTWERK Washing station (Cat.# 28050606/10-001)
- Light microscope with 20x objective and phase contrast (e.g. Ph1-0.4)

Reagents and Supplies

Component	Storage
BD Vacutainer® CPT™ Cell Preparation Tubes with Sodium Citrate 8 ml (Europe: BD Cat.# 362782, US: BD Cat.# 362761)	Upright, at room temperature (RT)
BD Vacutainer® Safety-Lok™ Blood Collection Set (Europe: BD Cat.#367284, US: BD Cat.#367292)	RT
ZellSafe™ Cell Chips (Cat.# 28050606/01-010, provided in a ZellSafe™ box)	4°C/ 39°F
ZellSafe™ Box (Cat.# 28050606/10-002, for storage of ZellSafe™ chips)	4°C/ 39°F
ZellScanner ONE Buffer Kit (Cat.# 28050606/07-003) containing: ZELLKRAFTWERK wash buffer ZELLKRAFTWERK fixation buffer ZELLKRAFTWERK storage buffer	 RT RT 4°C/ 39°F

Preparation of Clinical Trial Support

Clinical bioanalysis requires 6 ADDITIONAL chips for quality control (QC) purposes. When enrolling the first subject in a trial, please draw blood into 3 additional CPT tubes, and prepare 6 QC chips in addition to the study chips. Please store unloaded and loaded ZellSafe chips in different refrigerators to avoid accidental confusion.

Procedure

NOTE: One 8 ml BD Vacutainer® CPT™ tube is sufficient to load two ZellSafe™ cell chips.

A. Blood collection

1. Prior to the experiment, the BD Vacutainer® CPT™ tubes containing sodium citrate should be stored at RT and properly labeled for patient identification purposes.
2. Draw approximately 6 ml of blood (minimum volume) directly into each 8 ml Vacutainer® CPT™ tube using the standard technique for BD Vacutainer Evacuated Blood Collection Tubes. Invert the tubes about 10 times.
3. Store the BD Vacutainer® CPT™ tubes upright at RT until centrifugation. For optimum results, these samples should be centrifuged within **2 hours** after blood collection.

B. PBMC isolation

1. Mix the BD Vacutainer® CPT™ tubes containing the blood samples immediately before the centrifugation by gently inverting the tubes **8 to 10 times. DO NOT SHAKE.**
2. Place the tubes on the outer edge of the swinging bucket, and centrifuge **(20 min; 1600 g; RT**) while keeping the brake ON (Acc. 9, Dec. 9). Note: You must use a centrifuge with an horizontal rotor (swinging bucket) and an adaptor that can accommodate 16x125 mm tubes.
3. After the centrifugation, the PBMCs are concentrated in a whitish layer just beneath the plasma layer (see Fig. 1).

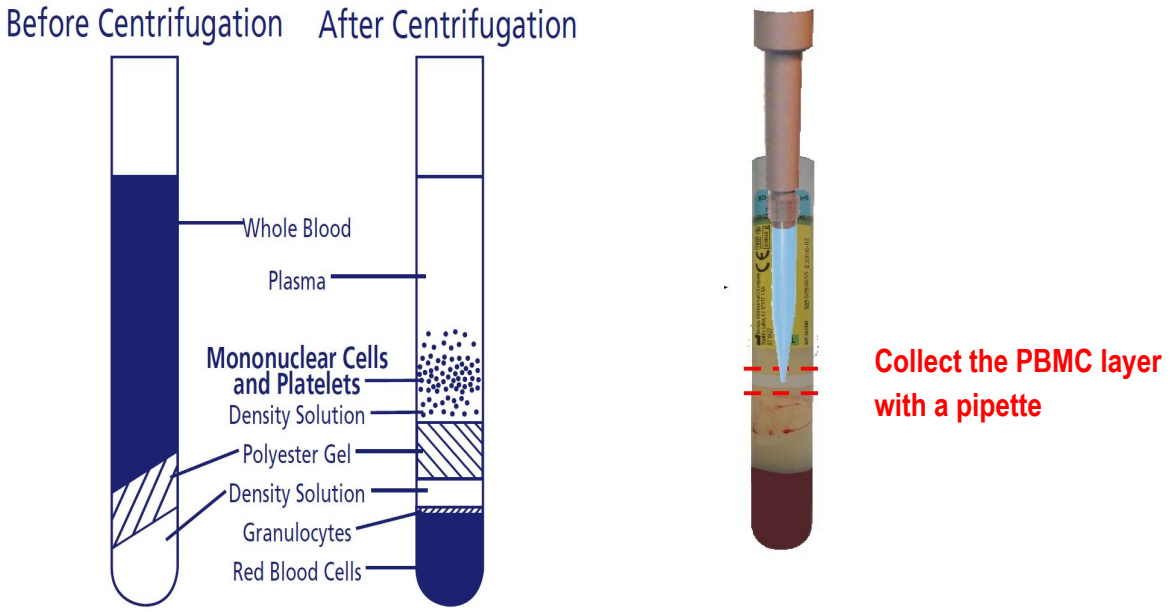

Fig. 1 | Position of the PBMCs after centrifugation (*Source: Manual BD Vacutainer® CPT™*)

4. Aspirate approximately half of the plasma using a 1000 µl pipette, without disturbing the mononuclear cell band (PBMC layer).
5. Carefully collect 3 times 200 µl of the PBMC layer with a 200 µl pipette, without touching the gel barrier, and transfer this fraction into a BD Falcon round-bottom tube with a cap. For optimum results, collect the PBMCs immediately after the centrifugation.
6. Add **1 ml ZELLKRAFTWERK wash buffer** and resuspend. Close the tube with the cap.
7. Place the tubes on the outer edge of the swinging bucket, and centrifuge (**5 min; 100 g; RT**) while keeping the brake ON (Acc. 9, Dec. 9). Do not centrifuge more than 5 min, or at a higher speed, because this causes extensive thrombocyte contamination.
8. Carefully remove and discard the supernatant (containing thrombocytes and debris) without disturbing the pellet. Resuspend the pellet in **1 ml ZELLKRAFTWERK wash buffer** while avoiding the formation of air bubbles.
9. Place the tubes on the outer edges of the swinging bucket, and centrifuge again (**5 min; 100 g; RT**) while keeping the brake ON (Acc. 9, Dec. 9).
10. Carefully remove and discard the entire supernatant without disturbing the pellet. Resuspend the pellet in **200 µl ZELLKRAFTWERK wash buffer**.

C. Preparation and loading of the ZellSafe™ chips

1. Apply the patient identification label on the ZellSafe™ chip at the position indicated in Fig. 2a (optional; not included in the kit). Please do not write on the QR-code label.
2. Place the chip with label side up in the ZELLKRAFTWERK Washing station. Remove the sealing plug from the inlet of the ZellSafe™ chip (Fig. 2b; do not discard the plugs!). Pipette a few drops of **ZELLKRAFTWERK wash buffer** into the inlet to remove the air.
3. Plug the pipette adapter into the inlet of the ZellSafe™ chip (Fig. 2c), and fill the adapter with **ZELLKRAFTWERK wash buffer**. Remove any air bubble from the pipette adapter by carefully aspirating the fluid.
4. Remove the sealing plug from the ZellSafe™ chip outlet. Rinse the chip with 3x 200 µl **ZELLKRAFTWERK wash buffer**. Make sure that all air bubbles are removed and that a flow is established before loading the ZellSafe™ chip with cell samples. Pipetting of all solutions (buffers and cell suspension) should be done drop-by-drop. The solutions flow through the chip by gravity. **NEVER LET THE CHIPS RUN DRY!**
5. Pipette 100 µl cell solution into the chip and allow the cells to settle **(5 min; RT)**.
6. Rinse the chip with 5x 200 µl **ZELLKRAFTWERK wash buffer** and **verify cell density** with a standard light microscope. Please refer to section *"D. Quality control"* on pages 7-8 for examples.
7. Rinse the chip with 5x 200 µl **ZELLKRAFTWERK fixation buffer**. Incubate **45 min at 4°C/ 39°F**.
8. Rinse the chip with 5x 200 µl **ZELLKRAFTWERK wash buffer**.
9. For storage rinse the chip with 5x 200 µl **ZELLKRAFTWERK storage buffer**.
10. Tightly seal the chip with the sealing plugs. First seal the outlet, thereafter the inlet.

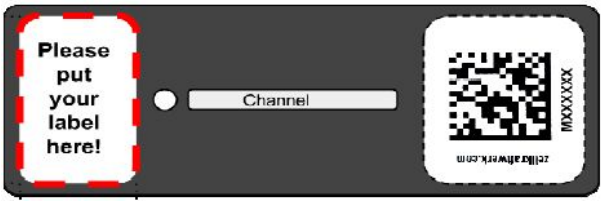

Fig. 2a | Space for additional label on the ZellSafe™ chip (label not included).

Fig. 2b | ZellSafe™ chip with sealing plugs.

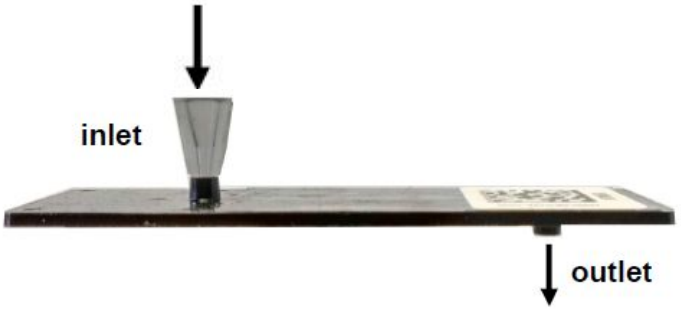

Fig. 2c | Zell Safe ™ chip with pipette adapter.

D. Quality control - cell density

After loading cells on the ZellSafe™ chips, please verify the quality and cell density with a standard light microscope. Examples of acceptable (Fig. 3a) and unacceptable (Fig. 3b) cell densities, and artefacts (Fig. 3c) are given below. If cell densities are too low, consult the “Troubleshooting“ section (page 9).

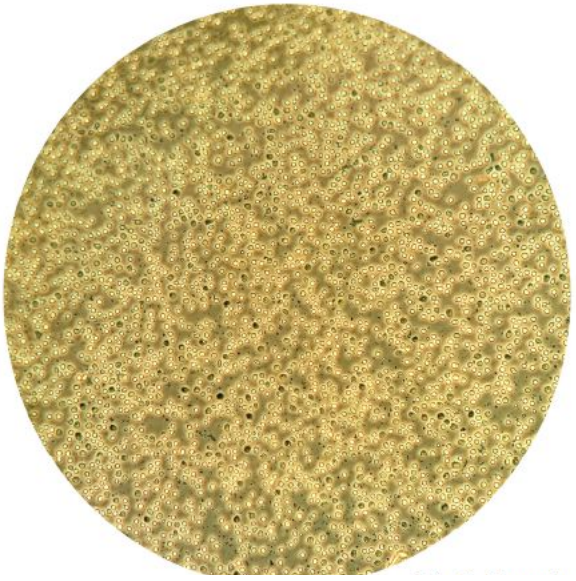

Fig. 3a | Example: accetable cell density (*200x*)

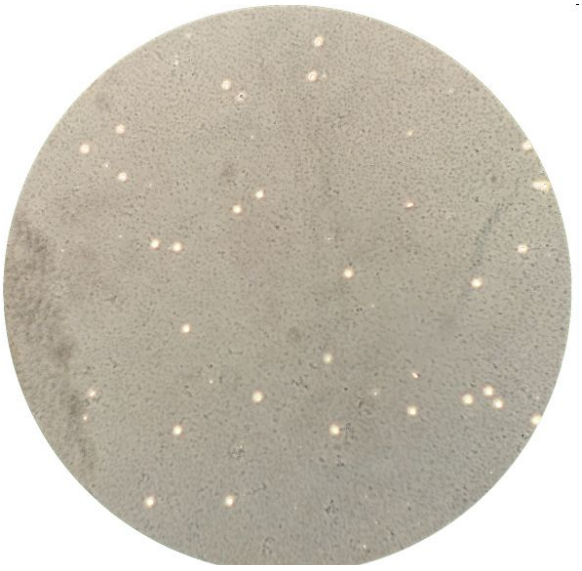

Fig. 3b | Example: unacceptable cell density (*200x*)

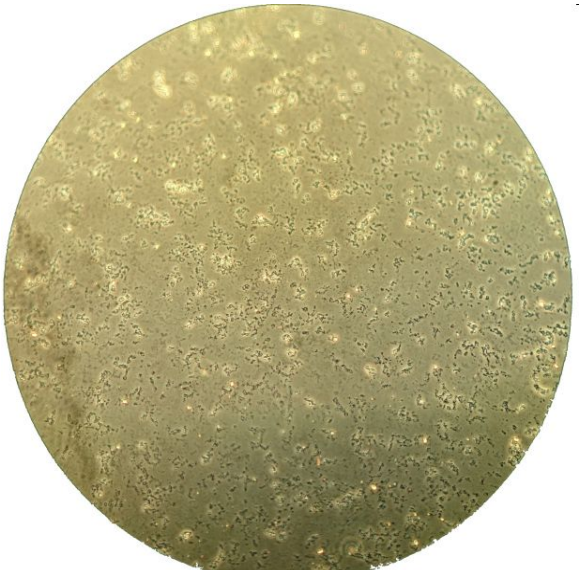

Fig. 3c | Example: Dirt, no cells (*200x*)

Note: The ZellSafe™ chips that are to be shipped should be stored in a ZellSafe™ box.
The shipping conditions are **4°C/ 39.2°F** with temperature tracking (RFID). **DO NOT FREEZE!**
Please complete the attached sample manifest and place it to the shipment.

Also, please ship the samples as described in detail in the **ZELLKRAFTWERK shipping instructions**.

E. Troubleshooting

Problem	Possible cause	Solution
No defined PBMC layer (Protocol step **B.3.**)	Incorrect adapter size	Use 16x125 mm centrifuge tube ada
	Centrifuge not calibrated	Calibrate correctly.
	Centrifugation speed too low	Increase speed to 1600 g.
	Centrifugation time too short	Increase centrifugation time (up to 30 min).
No gel movement (Protocol step **B.3.**)	Centrifugation speed too low	Increase speed to 1600 g.
	Centrifuge temperature less than 18°C/ 64°F	Increase temperature to 18-25°C/ 64-77°F.
Air bubbles in the chip (Protocol step **C.3.**)	Air infiltration	Carefully aspirate the fluid or pipette with slight pressure. As soon as cells are loaded pressure should be avoided.
No flow through the chip (Protocol step **C.4.**)	Clogged pipette adapter	Replace pipette adapter.
	Trapped air	Carefully aspirate the fluid or pipette with slight pressure. As soon as the cells are loaded, pressure should be avoided.
Low cell count on the chip (Protocol step **C.5.**)	Low cell count	If possible, reload the chip with 100 µl of the same sample **BEFORE** fixation.

Appendix 1:
Delivery Data Sheet / Sample Manifest

For ZellSafe chips that are to be shipped, please complete this data sheet and place it to the shipment. The Operator ID can be found on your ZELLKRAFTWERK Certificate.

Chip ID	Sample ID	Sample type, Description of samples	Storage conditions	Operator ID	To be completed by ZELLKRAFTWERK
M				**H**	
M				**H**	
M				**H**	
M				**H**	
M				**H**	
M				**H**	
M				**H**	
M				**H**	
M				**H**	
M				**H**	
M				**H**	
M				**H**	
M				**H**	
All items checked for amount and description: Name and Signature (to be signed by Zellkraftwerk)					
All items stored according to storage information: Name and Signature (to be signed by Zellkraftwerk)					

Please provide a phone number and a contact person in case of emergency.

Name: Phone number:

Please note that ZELLKRAFTWERK will not accept the delivery for quality assurance purposes if the delivered items do not match the description.