APPLICATION NOTE No. 401

EU-IVD Products

Faster PBMC-Based Diagnostic Using the Eppendorf Multipurpose Benchtop Centrifuges 5920 R and 5910 Ri

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Abstract

With their exceptional capacity, high flexibility and speed, the members of the new Centrifuges 59xx family bridge a gap between traditional benchtop centrifuges and floorstanding centrifuges. Their impressive variety of applications offers a number of advantages in diverse areas of clinical diagnostics including, for example, the isolation of peripheral blood mononuclear cells (PBMC). This method is based on the application of Ficoll-Paque® density gradient centrifugation in 15/50 mL conical tubes, or blood collection tubes, respectively. Obtaining clean, well-separated PBMCs, and thus a maximum yield of viable cells, is essential in keeping sample loss to a minimum. The Multipurpose Centrifuge 5920 R* as well as 5910 Ri* delivered exceptional results for this application, even at acceleration/deceleration rates of 9/3.

CE * The Centrifuges 5920 R and 5910 R are in-vitro diagnostic devices according to Directive 98/79/EC of the European Parliament and the Council dated October 27, 1998.

Figure 1: Centrifuge 5910 Ri with Rotor S-4xUniversal

Introduction

Human blood consists of equal parts of blood plasma and blood cells. These include erythrocytes (red blood cells), leukocytes (white blood cells) and thrombocytes (platelets). Leukocytes are further subdivided into different cell types. These include lymphocytes and monocytes, which (in cooperation with other cells) form the basis of the innate immune system and which, owing to their single nucleus, are referred to as peripheral blood mononuclear cells (PBMC).

The term lymphocyte encompasses two major classes, B-lymphocytes and T-lymphocytes. B-lymphocytes are responsible for antibody production, whereas T-lymphocytes produce signal molecules which will ultimately lead to the removal of diseased or foreign cells.[1]

Lymphocytes are isolated from "buffy coats" (whole blood concentrates without serum). PBMCs can be separated from other components of the blood, such as erythrocytes and granulocytes, via density gradient centrifugation using Ficoll-Paque PLUS. Ficoll has a density of 1.007 g/mL. Due to their higher density, erythrocytes, granulocytes and dead cells will pass through the Ficoll layer, whereas lymphocytes and monocytes, based on their lower density, will accumulate at the plasma-gradient boundary (figure 2). This approach is concordant with the method for isolation of PBMC, developed by Bøyum in 1968. [2] [3]
Today, countless applications in biomedical routine diagnostics rely on highly viable, functionally intact cell populations. Due to its uncomplicated and robust feasibility, density gradient centrifugation is now ubiquitously applied worldwide. One prerequisite for the successful isolation of PBMC is the formation of a defined interphase. To this end, the entire procedure must be carried out with a minimum of vibration. Generally, a mixing of the phases can only be avoided if the rotor brake is deactivated [4] – a measure which constitutes an extremely time-consuming step within this application. This Application Note will show that the new Eppendorf Multipurpose Centrifuges 5920 R and 5910 Ri, in combination with a variety of swing-out rotors, are capable of meeting such high demands and how at the same time the user will benefit from considerable time savings afforded by the individual selection of acceleration and deceleration ramps.

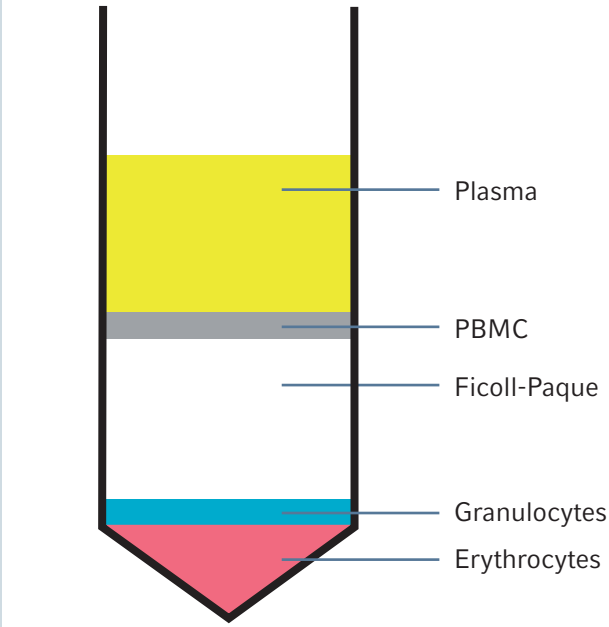

Figure 2: Density gradient of a buffy coat with Ficoll-Paque PLUS (schematic)

Materials and Methods

Methods

Ficoll-Paque PLUS density gradient centrifugation

1. Bring Ficoll-Paque PLUS and PBS buffer to room temperature.
2. Invert Ficoll-Paque PLUS several times.
3. Wipe the blood pouch with 70% ethanol, cut the lower tube and transfer the blood to a wide-mouth flask in a sterile manner.
4. Dilute the blood 1:1 in PBS, close the bottle and mix by careful inversion.
5. Place 15 mL of Ficoll-Paque into each of the 50 mL Conical Tubes.
6. Overlay the Ficoll-Paque with the blood/PBS mixture using a serological pipette at the lowest speed setting.
   TIP: In order to avoid compromising the subsequent purity of the PBMC, the mixing of Ficoll-Paque and blood must be avoided at all cost. For this reason, the tube is best held at an angle, and in order to achieve a good overlay, the blood mixture should be released slowly from the pipette, touching the tube wall.
7. Centrifuge the sample in the desired swing-out rotor at 400 x *g* and 20 °C for 30 min, selecting acceleration/deceleration rates of 9/0 or 9/3*, respectively, at the setting "at set rpm".
   TIP: Typically, the rotor brakes must be inactivated entirely in order to effectively prevent mixing of the phases. Strict adherence to temperature specifications is equally important as differences in temperature will change the density ratios of the liquids and may therefore negatively impact the results of the separation.
8. After completing the centrifugation, carefully remove the samples from the centrifuge to avoid mixing of the phases.

*Possible ramp settings Centrifuge 5920 R/5910 Ri: deactivated acceleration and deceleration (0/0), up to fastest acceleration and brake (9/9).

Purification of lymphocytes

1. Carefully aspirate 2/3 of the top layer (containing the plasma and platelets) using a sterile serological pipette until the interphase (containing the mononuclear cells) is within reach.
2. Using an Eppendorf Research® plus pipette*, aspirate the entire lymphocyte layer, while keeping the volume to a minimum, and transfer to a fresh tube.
   TIP: During this step, care should be taken to transfer as little Ficoll-Paque PLUS and supernatant as possible.
3. Add at least 3 volumes of PBS to the lymphocyte layer and carefully mix by pipetting up and down.
4. Centrifuge at 100 x *g* and 20 °C for 10 min and discard the supernatant.
5. Repeat steps 3 and 4.
6. Resuspend the cell pellet in a medium suitable for down stream applications.

Viability testing and determination of yield

1. Dilute the cells 1:1 in trypan blue and then either perform a manual cell count or analyze the cells using an automated cell counter.
2. Determine viability and yield.

Materials

Eppendorf Multipurpose Benchtop Centrifuge 5920 R with the following swing-out rotors:

1. Rotor S-4xUniversal-Large plus adapter 50 mL Conical Tubes
2. Rotor S-4x1000 with high capacity buckets plus adapter 50 mL Conical Tubes
3. Rotor S-4x750 plus adapter 50 mL Conical Tubes (not shown)

Eppendorf Multipurpose Benchtop Centrifuge 5910 Ri with the following swing-out rotors:

1. Rotor S-4xUniversal plus adapter 50 mL Conical Tubes (not shown)
2. Rotor S-4x750 plus adapter 50 mL Conical Tubes (not shown)
3. Rotor S-4x500 plus adapter 50 mL Conical Tubes (not shown)

> Conical Tubes 50 mL
> Eppendorf Research® plus Pipette 1000 µL, Eppendorf
> Electronic pipetting aid for serological pipettes
> Ficoll-Paque PLUS, GE Healthcare Bio-Sciences AB
> Dulbecco's Phosphate Buffered Saline 1x(DPBS), gibco® by Life Technologies™, Thermo Fisher Scientific®
> Buffy coats, human, University Clinic Eppendorf, Institute for Transfusion Medicine, tested negative for infectious diseases and Herpes viruses (from the previous day)
> Serological pipettes 25 mL
> Wide mouth flask 400 mL
> Surface disinfectant Bacillol® plus, Bode Chemie®
> Trypan blue 0.4%, SIGMA-ALDRICH®
> Microscope Axio Observer.A1, Zeiss®

CE * The Research plus pipette is an in vitro diagnostic device according to Directive 98/79/EC of the European Parliament and the Council dated October 27, 1998.

Results and Discussion

In order to evaluate the quality of the separation, it was determined whether a defined interphase with clearly delineated phase transitions was visible. Turbidity of the liquid may indicate suboptimal separation of the PBMCs. Figure 3 (a-d) shows the results obtained from the density gradient centrifugations carried out in the Centrifuge 5920 R using different swing-out rotors (the results obtained with the Centrifuge 5910 Ri were equivalent; not shown).

For each rotor, acceleration and deceleration rates of 9/0 and 9/3 were tested. It is evident that optimal separation of blood components was achieved in all cases and that vortex effects during deceleration of the rotor were largely avoided. This result is especially striking when compared to other centrifuges which yielded less impressive results, most likely due to rotor vibration during the run (figure 3e).

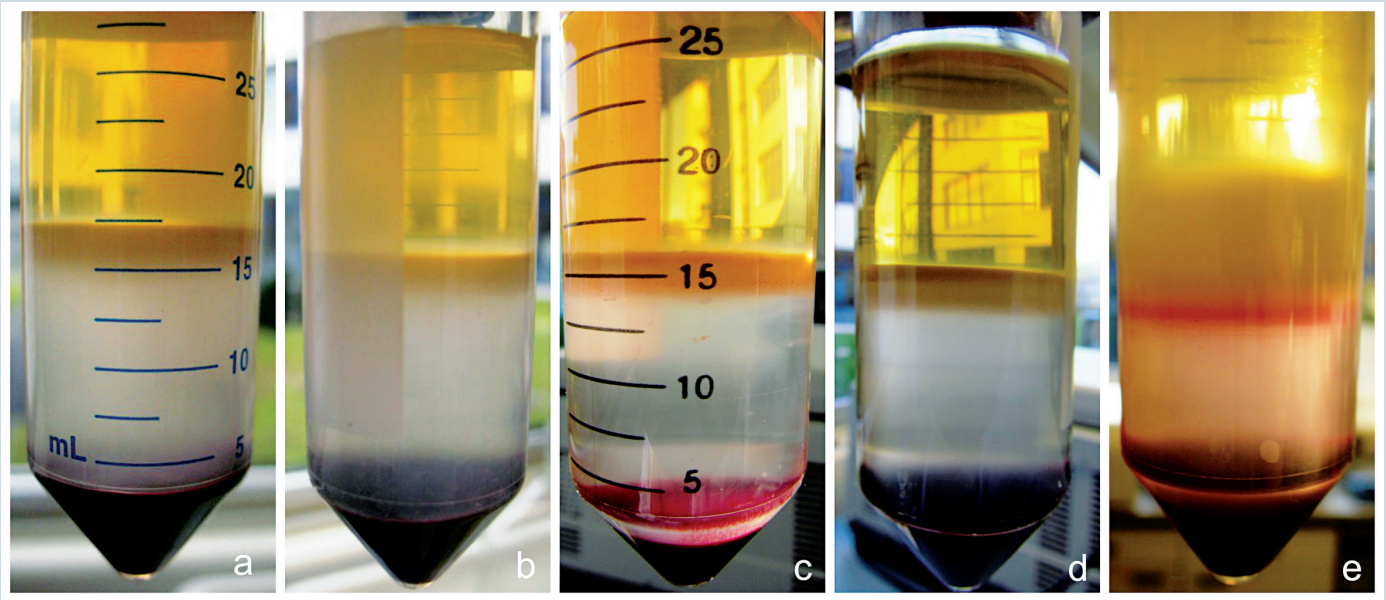

Figure 3: Results obtained after density gradient centrifugation in the Centrifuge 5920 R.
a) Rotor S-4x1000 with high capacity buckets (ramp 9/0)
b) Rotor S-4x1000 with HC buckets (ramp 9/3)
c) Rotor S-4xUniversal-Large (ramp 9/0)
d) Rotor S-4xUniversal-Large (ramp 9/3)
e) Negative example: cloudy interphase, turbid plasma/Ficoll phase.
(Results for the Centrifuge 5910 Ri not shown but identical)

In order to confirm the quality of the PBMC isolation using Ficoll-Paque PLUS, the PBMC samples obtained from the Rotor S-4xUniversal-Large were subjected to an analysis of their yield and viability, in addition to visual inspection. Data provided by GE Healthcare®, which routinely achieved a viability of 95% (+/-5%) during internal testing, served as a reference. [5]The studies carried out at Eppendorf showed an average viability of 94%, which is in line with expected values.
According to the literature, the expected yield for mononuclear cells falls between 0.8 and 3.2 x 10^6 cells/mL of blood.[6] Therefore, results of 3.0 x 10^6 cells/mL buffy coat (ramp 9/0) and 2.16 x 10^6 cells/mL buffy coat (ramp 9/3) also place the total yield of viable cells within a very good range.
These results demonstrate that the Centrifuges 5920 R and 5910 Ri fully meet the demands of low vibration performance, independent of the swing-out rotor used, and independent of whether an acceleration/ deceleration rate of 9/0 or 9/3 was selected. Furthermore, a ramp of 9/3 affords considerable time savings of up to 23.8 minutes (88%) as compared to the centrifugation parameters recommended in the literature (deactivated brake) (figures 4 and 5).[7]

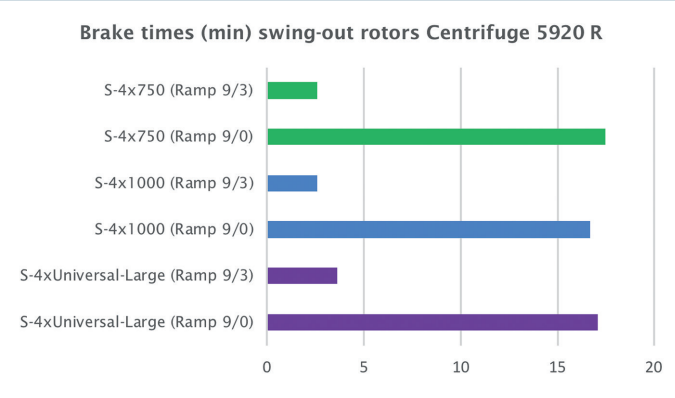

Figure 4: Brake times determined for the new Eppendorf Multipurpose Centrifuge 5920 R, used in combination with different swing-out rotors at ramp settings of either 9/0 or 9/3, respectively (rotors were fully loaded; the brake times decrease with decreasing load).

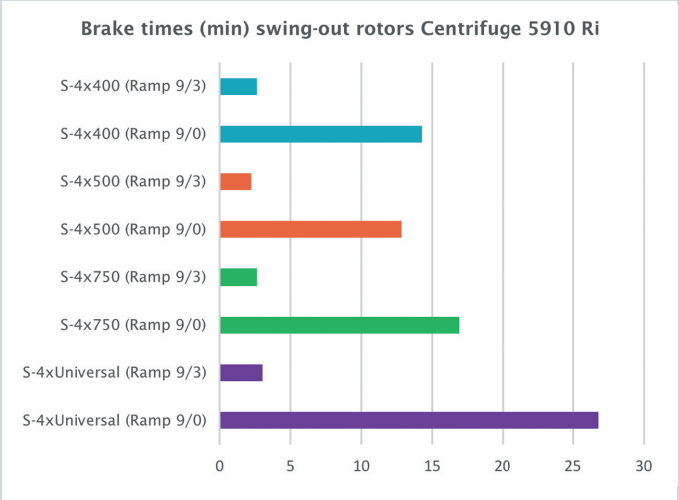

Figure 5: Brake times determined for the new Eppendorf Multipurpose Centrifuge 5910 Ri, used in combination with different swing-out rotors at ramp settings of either 9/0 or 9/3, respectively (rotors were fully loaded; the brake times decrease with decreasing load).

Conclusion

Eppendorf recommends the new family of Multipurpose Benchtop Centrifuges 59xx for density gradient centrifugation using Ficoll-Paque PLUS for the purpose of obtaining high yields of viable PBMCs for diagnostic purposes. According to current data, a consistently high quality of results can be expected for all available swing-out rotors. Owing to their large capacity, paired with the ability of significantly reducing the time required for rotor deceleration, both Centrifuges are very well suited for diagnostic laboratories processing high sample volumes.

Literature

[1] Kleiveland C.R. (2015). Peripheral Blood mononuclear cells. In: Verhoeckx K. et al. (eds) The Impact of Food Bioactives on Health. Springer, Cham
[2] Bøyum A. Isolation of mononuclear cells and granulocytes from human blood. Scan. J. Clin. Lab. Invest. 1968, 21 Suppl. 97): 77-89
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[4] Heine H, Uschkureit T. Software-controlled acceleration and deceleration rates for optimal isolation of mononuclear cells. Eppendorf Application Note 74; www.eppendorf.com
[5] Ficoll-PaqueTMplus Instructions. www.gelifesciences.com
[6] HIV/AIDS Network Coordination: Cross-Network PBMC Processing Standard Operating Procedure. www.hanc.info
[7] Luttman W, Bratke K, Küpper M, Myrtek D. Der Experimentator: Immunologie. 4th edition, Heidelberg: Springer Publishers; 2014

Ordering information

Description	Order no.
Centrifuge 5920 R (EU-IVD), refrigerated, key pad, 230 V/50 – 60 Hz	5948 000 018
Rotor S-4xUniversal-Large, incl. universal buckets	5895 190 006
Rotor S-4x1000, incl. high-capacity buckets	5895 118 003
Rotor S-4x750, incl. 4x750 mL round buckets	5895 120 008
Centrifuge 5910 Ri (EU - IVD), refrigerated, touchscreen interface, 230 V/50 – 60 Hz	5943 000 017
Rotor S-4xUniversal, incl. universal buckets	5895 200 001
Rotor S-4x750, aerosol-tight, without buckets	5895 121 004
Rotor S-4x750, aerosol-tight, incl. 4 x 750 mL round buckets	5895 120 008
Rotor S-4x500, incl. 4 rectangular buckets	5895 170 005
Rotor S-4x400, incl. 4 round buckets	5895 180 000
Eppendorf Research® plus, Single channel, variable, 100 – 1000 µL, blue	3120 000 062

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