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Platelet removal by single-step centrifugation

Linda G. Rikkert[1,2,3], Frank A. W. Coumans[3,4], Chi M. Hau[2,3], Leon W. M. M. Terstappen[1], & Rienk Nieuwland[2,3]

[1]Medical Cell BioPhysics, University of Twente, Enschede, The Netherlands, [2]Amsterdam UMC, University of Amsterdam, Laboratory of Experimental Clinical Chemistry, Amsterdam, The Netherlands, [3]Amsterdam UMC, University of Amsterdam, Vesicle Observation Center, Amsterdam, The Netherlands, and [4]Amsterdam UMC, University of Amsterdam, Biomedical Engineering and Physics, Amsterdam, The Netherlands

Abstract

The study of extracellular vesicles (EVs) in plasma requires removal of cells including platelets. At present, a two-step centrifugation protocol is recommended and commonly used. A simpler protocol that is less operator dependent is likely to improve the quality of plasma samples collected for EV research. The objective of this study is to develop an easy, fast and clinically applicable centrifugation protocol to produce essentially platelet-free plasma with a high yield for EV research. We compared the two-step centrifugation protocol to a single-step protocol at 5,000 *g* for 20 minutes. The removal of platelets was computationally predicted and experimentally validated. Flow cytometry was used to detect residual platelets and platelet-derived (CD61+) EVs. The single-step protocol at 5,000 *g* (i) is less laborious and approximately ten minutes faster, (ii) removes platelets as effective as the two-step centrifugation protocol, and (iii) has a ~ 10% higher plasma yield, whereas (iv) the recovery of platelet-derived EVs is comparable. For future research on plasma EVs we recommend the newly developed, easy and fast single-step protocol for preparation of platelet-free plasma for research on plasma biomarkers including EVs.

Keywords

Blood, blood platelets, centrifugation, extracellular vesicles, flow cytometry

History

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Introduction

Extracellular vesicles (EVs) are cell-derived nanoparticles that are abundantly present in body fluids like plasma. Plasma EVs have an outstanding biomarker potential, but studying such EVs requires removal of platelets during blood collection and handling [1]. In the most applied protocol, blood is centrifuged at 2,500 *g* for 15 minutes to obtain platelet-poor plasma. Subsequently, the platelet-poor plasma is transferred to a new centrifugation tube and centrifuged at 2,500 *g* for 15 minutes to obtain essentially platelet-free plasma as shown in Figure 1 [2]. This protocol was developed to optimize platelet removal, thereby improving the comparison of EV measurement results, and therefore this protocol was included in recent guidelines [3,4]. Because this protocol includes two centrifugation steps, this protocol is more time-consuming to perform than a single-step centrifugation protocol. A simpler protocol that is less operator dependent is likely to improve the quality of plasma samples collected for EV research.

Previously, we developed a model to study how centrifugation protocols influence the purity of cancer biomarkers in blood [5]. These biomarkers included circulating tumor cells, (tumor-educated) platelets, tumor-derived EVs, EV-miRNAs, and circulating cell-free DNA. In the present study, we applied this model to develop and validate an easier and faster protocol to prepare essentially platelet-free plasma without the loss of EVs.

Correspondence: Rienk Nieuwland, Amsterdam UMC, Laboratory of Experimental Clinical Chemistry, Vesicle Observation Center, University of Amsterdam, Amsterdam, AZ 1105, The Netherlands. E-mail: r.nieuwland@amsterdamumc.nl
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Methods

Stokes Model

We computed the protocol using the previously described model [5]. Briefly, the Stokes equation describes the distance a particle travels through a medium, which depends on the diameter of the particle, volumetric mass density of the particle and medium, viscosity of the medium, time of centrifugation, and gravitational acceleration of the rotor. Centrifugation of particles that are uniformly distributed throughout the sample causes large, high density particles like cells to move down to the pellet (Figure 1 below the dashed line), while small, low density particles, like EVs stay in the supernatant (above the dashed line). The variables required to compute the model output were applied as described previously [5] (see Table I). Importantly, platelets were modeled as oblate spheroids with a density ranging from 1.05–1.09 g/mL, independent of platelet size. EVs were modeled as spheres, with a size dependent density ranging from 1.10 g/mL for 100 nm diameter down to 1.06 g/mL for 1 μm diameter.

Model Validation

Blood was obtained from ten healthy donors with informed consent in accordance with the Declaration of Helsinki and the study protocol was waived by the medical ethics committee of the Amsterdam University Medical Center. Whole blood was drawn using a 21 G needle. The first 3.5 mL was discarded. For each of the ten donors, nine 3.2% citrate blood collection tubes of 3.5 mL (Greiner Bio-one, Kremsmünster, Austria) were collected, mixed

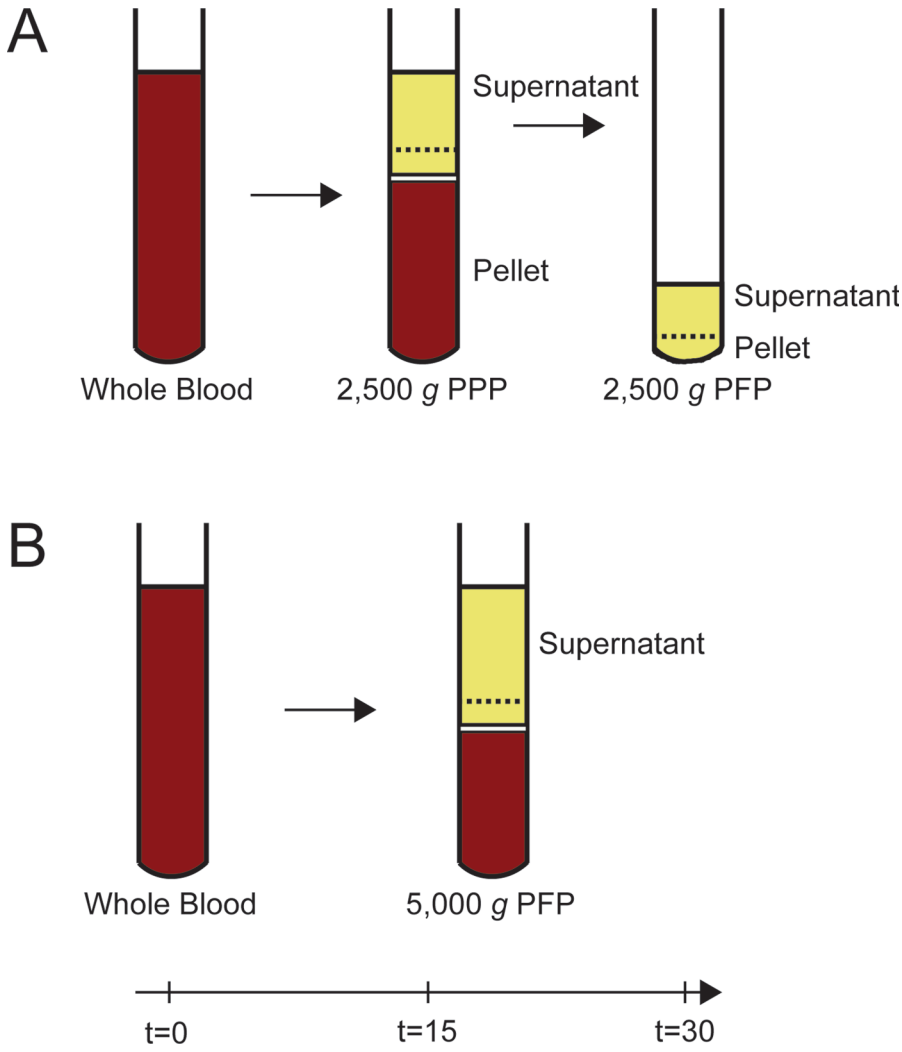

Figure 1. Centrifugation protocols to remove platelets from whole blood. A) Using a commonly applied protocol, platelet poor plasma (PPP) is obtained by centrifugation of whole blood at 2,500 *g* for 15 minutes which will deplete cells and part of the platelets. A second centrifugation step at 2,500 *g* for 15 minutes of the supernatant depletes most of the residual platelets (PFP, platelet-free plasma). B) Using the single-step centrifugation protocol, PFP is obtained by centrifugation of whole blood at 5,000 *g* for 20 minutes.

Table I. **Model variables and their net effect on particle velocity**. A particle in a gravitational field accelerates due to the gravitational force and decelerates due to viscous drag. From a velocity of zero, a particle accelerates due to gravity and has zero deceleration due to drag. As a particle accelerates, the drag force increases until the particle reaches a velocity where the drag force equals and thus balances the gravitational force. The Stokes equation describes the velocity at which the two forces are equal.

Variable	Definition (units)	Impact of parameter increase	Net effect on particle velocity
$\Delta\rho$	Difference between particle and medium density (kg/m^3)	Acceleration due to increase in net particle mass	Increases
d	Particle diameter (m)	Acceleration increases due to increase in net particle mass Drag increases	Increases
g	Gravitational acceleration (m/s^2)	Acceleration increases due to increase in gravity	Increases
S	Shape factor, sphere = 1	Increased drag	Decreases
t	Time (s)	Travel distance increases	None
v	Velocity (m/s)	-	-
η	Viscosity (kg/m·s)	Increases drag	Decreases

gently with the anti-coagulant and processed within 15 minutes. Seven blood collection tubes were centrifuged at 2,500 *g* for 15 minutes using a Rotina 380 R centrifuge (Hettich, Tuttlingen, Germany) at 20°C without brake (see Figure 1). Platelet poor plasma was collected with a plastic Pasteur pipette (VWR, Radnor, PA), leaving 1 cm of plasma above the buffy coat and taking care not to disturb the buffy coat to circumvent cell contamination. Next, the approximately 9 mL of platelet poor plasma was pooled.

For the original two-step protocol, 3.5 mL of platelet poor plasma was transferred to a conical base tube (10 mL; Sarstedt, Nümbrecht, Germany). The tube was centrifuged a second time at 2,500 *g* for 15 minutes at 20°C without brake. After centrifugation, platelet-free plasma was collected using a P1000 pipette leaving 100 uL pellet as described in the original description of the two-step protocol [2]. For the conservative two-step protocol, another tube containing 3.5 mL of platelet poor plasma was centrifuged a second time at 2,500 *g* for 15 minutes at 20°C without brake. After centrifugation, platelet-free plasma was collected 1 cm above the bottom of the tube using a Pasteur pipette. For the single-step protocol, one blood collection tube was centrifuged at 5,000 *g* for 20 minutes using a Rotina 420 R centrifuge (Hettich, Tuttlingen, Germany) at 20°C without brake, and platelet-free plasma was collected leaving 1 cm of plasma above the buffy coat using a Pasteur pipette. The remaining tube was used to measure the platelet concentration in whole blood.

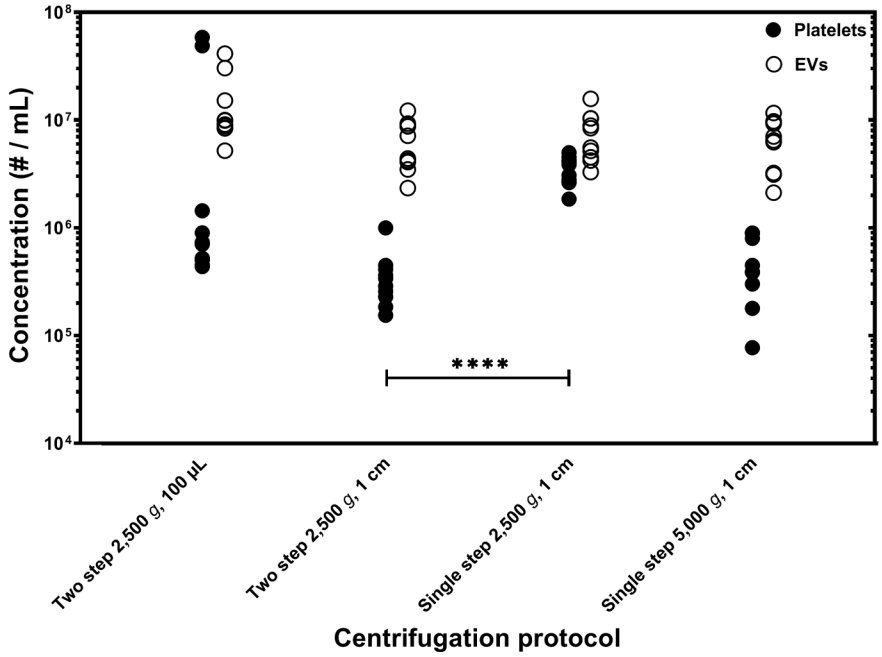

Figure 2. Concentrations of residual platelets and platelet-derived extracellular vesicles (EVs) after centrifugation. The original two-step 2,500 *g* protocol, 100 µL results in an increased concentration of residual platelets for two of the measurements compared to the other centrifugation protocols. Leaving 1 cm of plasma above the pellet (the conservative two-step protocol) instead of the original (100 µL) protocol results in a comparable residual platelet concentration. A single centrifugation step at 2,500 *g* for 15 minutes is less effective in removing platelets compared to the two-step 1 cm centrifugation protocol ($p < .0001$, ****). Replacing the two-step centrifugation protocol by a single 5,000 *g* step results in a comparable residual platelet concentration compared to the original two-step centrifugation protocol. The EV concentration (open symbols) is similar for the two-step centrifugation protocol and single 5,000 *g* step protocol, suggesting that no EVs are lost at a higher speed and longer centrifugation time.

Flow Cytometry

Because antibody aggregates can be detected as EVs using flow cytometry, antibody aggregates were removed by centrifugation at 18,890 *g* for 5 minutes at 20°C. To measure the concentration of platelet and platelet-derived EV concentration by flow cytometry, 20 µL of each fraction was incubated with 2.5 µL anti-CD61-APC (Y2/51, final concentration 50 µg/mL; Dako Denmark, Glostrup, Denmark), for two hours at room temperature in the dark. The labeling was stopped by adding 200 µL phosphate buffered saline (PBS; 21–031-CV; Corning, Corning, NY) to the samples. Samples were pre-diluted in PBS if necessary to event rates below 5,000/s to prevent swarm when triggering on side scatter [6].

We performed flow cytometry measurements on an A60-Micro (Apogee; Northwood, UK) at a flow rate of 3.0 µL/minute. Samples were measured for 1.5 minutes triggered on 405-nm side scatter. The side scatter trigger threshold corresponds to a side scattering cross section of 10 nm^2 (Rosetta Calibration; Exometry, Amsterdam, The Netherlands). Both platelets and EVs were identified by CD61-APC signal exceeding background fluorescence in the unstained sample (sample without antibody). Based on side scatter, platelets and EVs can be distinguished. Concentrations in this manuscript are the number of detected particles multiplied by the total sample dilution divided by flow rate and measurement time. Data were analyzed using FlowJo (v10, FlowJo, Ashland, OR) and Prism 7.0 (GraphPad, La Jolla, CA). Statistical analysis was performed using Prism 7.0. We applied the student's t-test to see if a statistical difference in residual platelet concentration was found between the centrifugation protocols.

Table II. Obtained plasma yield for each centrifugation protocol.

Protocol	Obtained plasma (mL)	Plasma yield (plasma/3.5 mL blood %)
Two step 2,500 *g*, 100 µL	1.0	29
Two step 2,500 *g*, 1 cm	0.9	26
Single step 2,500 *g*, 1 cm	1.1	31
Single step 5,000 *g*, 1 cm	1.2	34

Results and Discussion

The Stokes model suggests that the efficacy of platelet removal by a single-step centrifugation step at 5,000 *g* for 20 minutes should be comparable to the original two-step centrifugation protocol without affecting the concentration of EVs. Based on the model the expected percentage of EVs (200 nm – 1 um) left after centrifugation are 37.8% for the original 100 µL protocol and 36.2% for the single 5,000 g centrifugation protocol in case of centrifugation of 2 mL of blood. These numbers are comparable. For both protocols the concentration of platelets is below 0.02% after centrifugation. In this study, we show the efficacy of platelet removal is comparable between the single 5,000 *g* protocol and the original two-step centrifugation protocol without affecting the concentration of EVs for blood collection tubes up to 3.5 mL.

Figure 2 shows the measured concentrations of residual platelets and platelet-derived EVs for all centrifugation protocols. Left is the original two-step centrifugation protocol, in which approximately 100 µL of plasma is left above the pellet (see Figure 1) as described previously [2]. For this protocol disturbance of the pellet is expected because the plasma is collected near the (cell) pellet, which would increase the concentration of residual platelets in the supernatant. We observed indeed such an increase compared to the other two-step centrifugation protocol for two of the measurements (see Figure 2). Relative to the platelet concentration in blood (data not shown), median 0.5% (range 0.3–-23.6%) of platelets remain in the sample after the original two-step centrifugation protocol.

To minimize disturbance of the pellet, we also performed a 'conservative' two-step centrifugation protocol where we left about 1 cm of plasma above the pellet. This effectively reduces the variability of the residual platelet concentration compared to the original two-step centrifugation protocol. For the conservative

two-step protocol, median 0.1% (range 0.1–0.2%) of the platelets remain in the sample by this protocol. The yield of plasma with the conservative two-step centrifugation protocol is 26% compared to 29% for the original two-step centrifugation protocol, as shown in Table II.

A single centrifugation step for 15 minutes at 2,500 *g* is less effective in removing platelets compared to the two-step 1 cm centrifugation protocol ($p < .0001$, see Figure 2). Median 1.6% (0.7–2.0%) of the platelets remain in the sample by a single-step 2,500 *g* protocol.

If we replace the two-step centrifugation protocol by the model-based protocol, that is by increasing the centrifugation speed from 2,500 *g* to 5,000 *g* as well as the time from 15 minutes to 20 minutes, platelet removal is about as efficient as the two-step centrifugation when leaving 1 cm of plasma above the pellet. No significant difference ($p > .05$) between the protocols was found, but the plasma yield is ~10% higher compared to the original two-step centrifugation protocol, which implies that more plasma is collected and can be stored for biobanking. Median 0.1% (0–0.5%) of the platelets remain by this protocol. The concentration of platelet-derived EVs is comparable, suggesting that these EVs are not lost in the single 5,000 *g* protocol. Also the concentration of CD45 (leukocyte)- and CD235a (erythrocyte)-EVs (Figure S1 and S2) and the percentage of activated platelets (CD62p+) (Figure S3) are comparable between protocols. Furthermore, as this protocol includes only a single aspiration step performed well above the pellet, this protocol is less prone to variation between users.

The single 5,000 *g* centrifugation step is easier, faster, and sufficient in platelet removal from whole blood with a higher plasma yield compared to the original two-step centrifugation protocol. Therefore, we recommend the newly developed protocol for preparation of platelet-free plasma for research on plasma biomarkers including EVs. Alternatively, a modified two-step protocol, with removal of plasma to about 1 cm above the pellet instead of 100 µL, seems a good alternative when plasma is not the limiting factor. One has to bear in mind that we only studied blood collected from healthy subjects, and to which extent our data can be extrapolated to patient studies, needs further investigation.

Author Contributions

Conceptualization, L.R., F.C., L.T, and R.N.; Formal analysis, L.R.; Investigation, L.R. and C.H.; Project administration, L.R.; Resources, R.N.; Software, F.C.: Supervision, F.C., L.T., and R.N.; Validation, L. R. and C.H.; Visualization, L.R.; Writing—original draft preparation, L.R.; Writing—review and editing, F.C., L.T., and R.N.

Disclosure Statement

The authors declare that the research was carried out in the absence of any personal, professional or financial relationship that could potentially be construed as a conflict of interest.

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Supplementary Material

Supplemental data for this article can be accessed on the publisher's website.

ORCID

Leon W. M. M. Terstappen http://orcid.org/0000-0001-5944-3787

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