Separation of cells for respirometric studies: peripheral blood mononuclear cells and platelets

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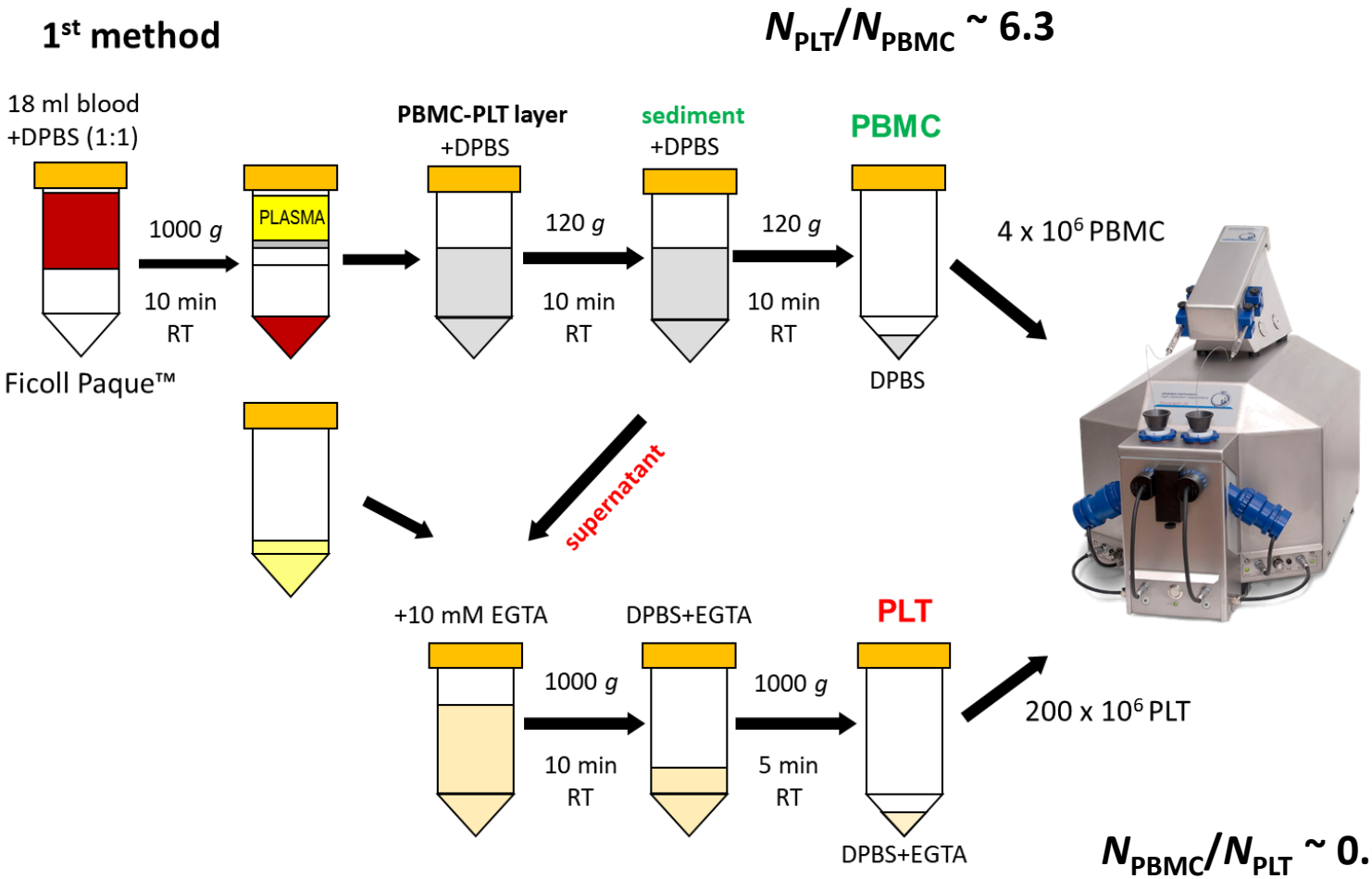
Isolation of PMBC and PLT from one blood sample

VACUETTE® K3EDTA tubes, 21 G needle

1st method: focus on PBMC RT

Mitochondrial Physiology Network
21.17(02):1-14 (2016)



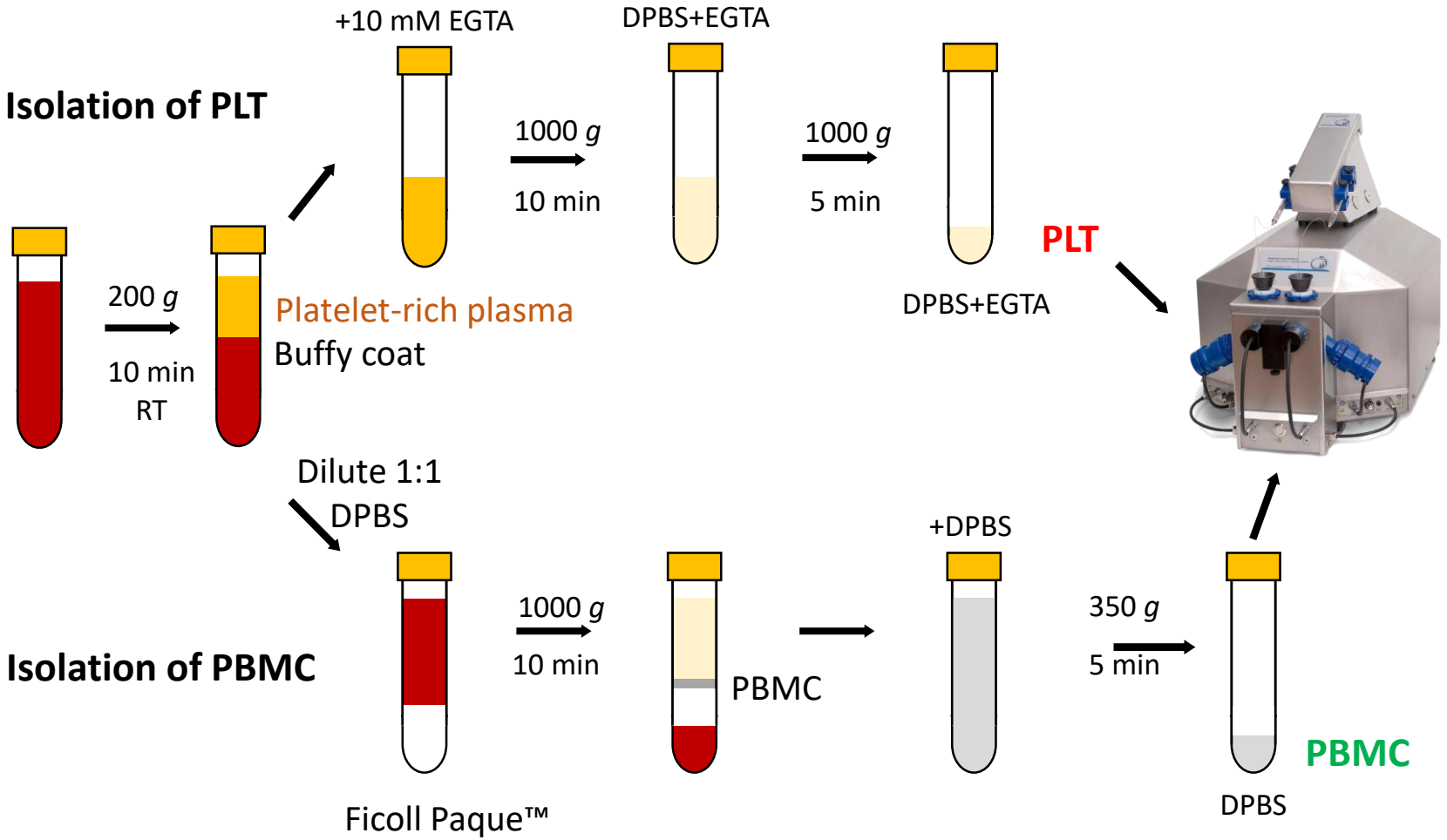
Isolation of PMBC and PLT from one blood sample

VACUETTE® K3EDTA tubes, 21 G needle

2nd method: focus on PLT

RT

Mitochondrial Physiology Network
21.17(02):1-14 (2016)

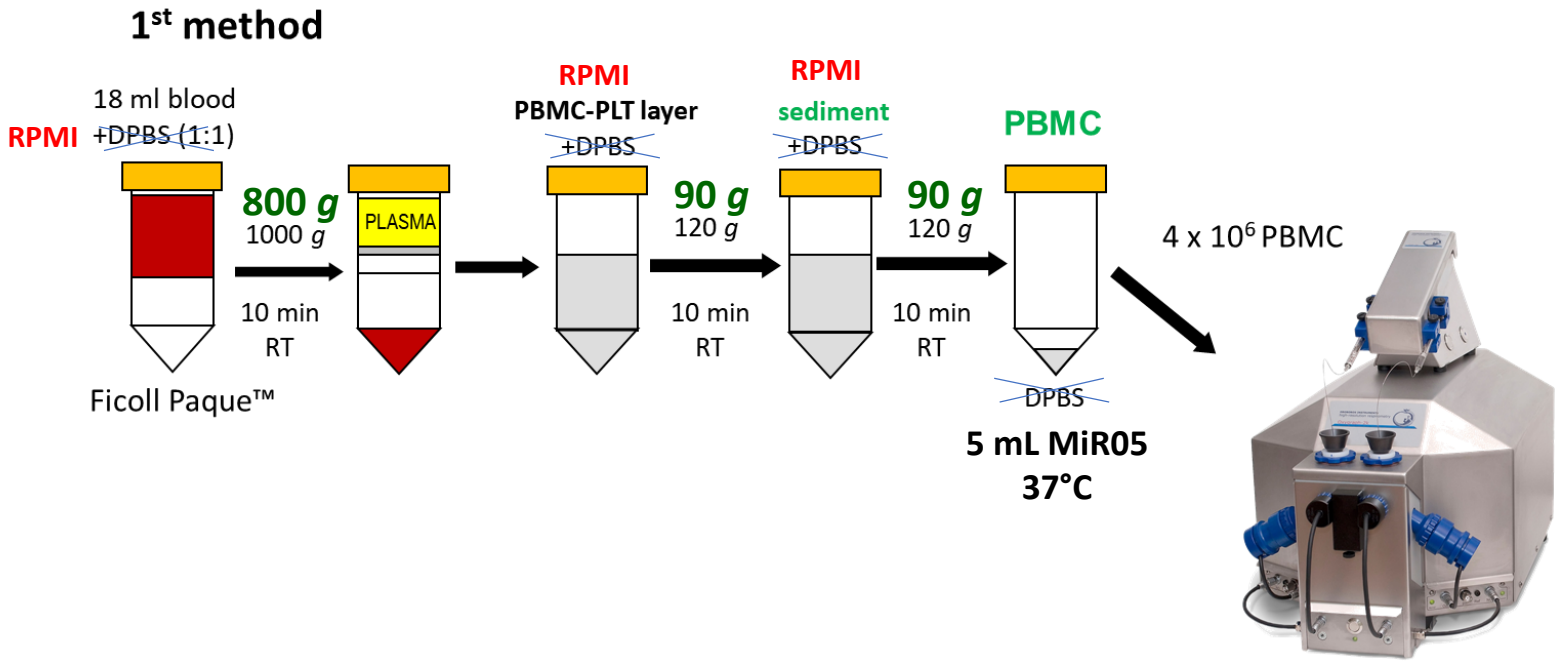


N_{PLT}/N_{PBMC} ~ 6

Verona method: isolation of PBMC

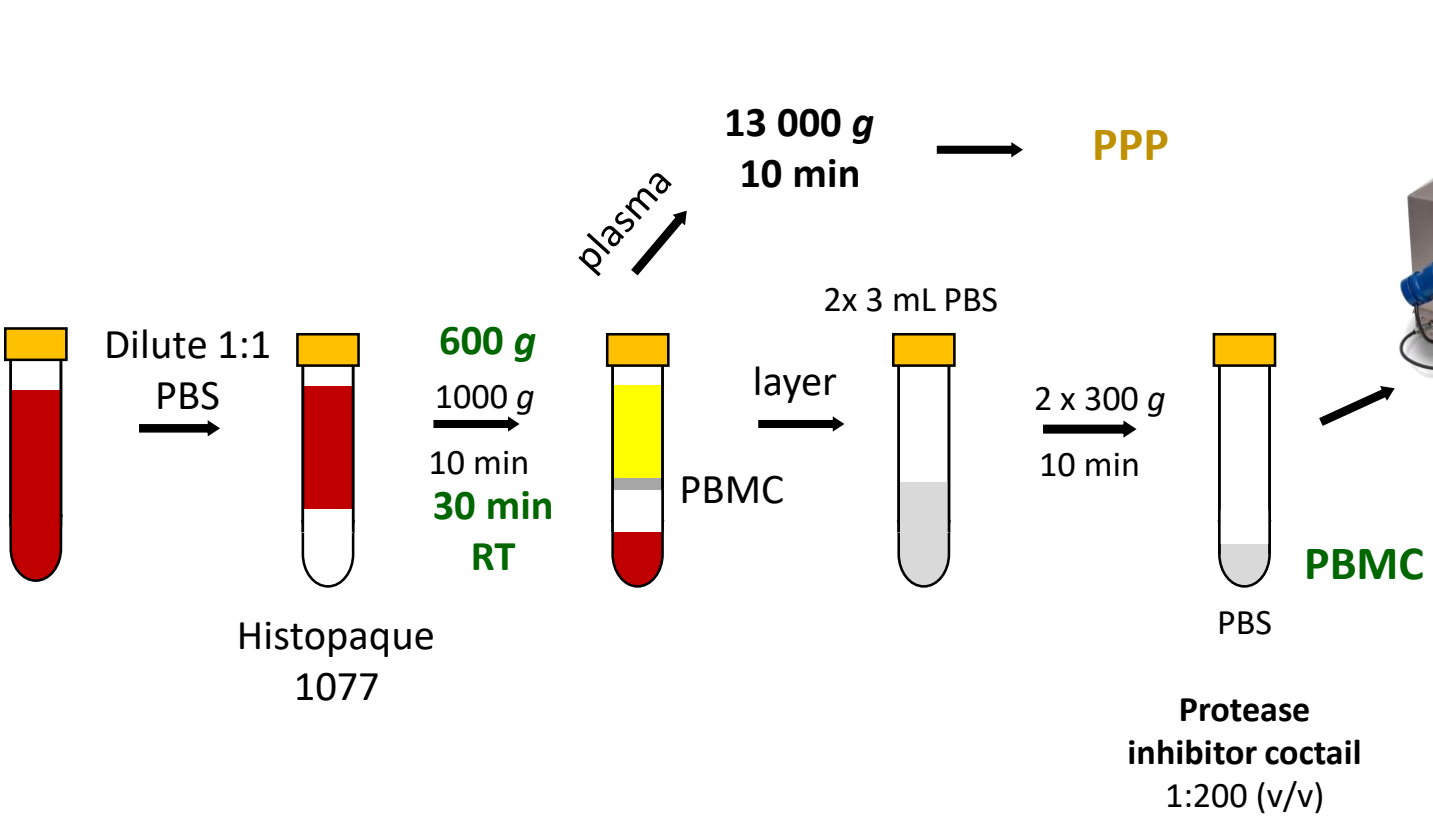
1st method: focus on PBMC

N_{PLT}/N_{PBMC} ~ 3.5



Poznan method: isolation of PBMC

Isolation of PBMC: N_{PLT}/N_{PBMC} ~ 0.03



Protocols for isolation of PBMC

Differences between protocols:

- centrifugation force – less PLT in the layer, lower recovery of PBMC
- centrifugation time => isolation time - ? effect
- washing media: DPBS, PBS – no substrates, no Ca^{2+}
  RPMI
- resuspension in MiR05 (37°C) vs (D)PBS – time ?

Outcome:

1. purity of PBMC fraction - count
2. ? effect on respiration

Differences between protocols for isolation of PBMC in the literature

- temperature: RT, 4°C

? effect on cell activation

- density centrifugation media: 1.077 g/mL

Lymphoprep	600 *g* 20 min, 20°C
Ficoll Paque Plus	
Ficoll Hypaque	900 *g*, 20 min, 25 °C
Histopaque	

? effect on cell composition of PBMC fraction:

lymphocytes, monocytes, neutrophils
PLT contamination

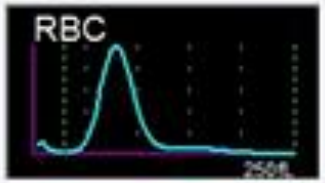
Sysmex XN-350 hematology analyzer

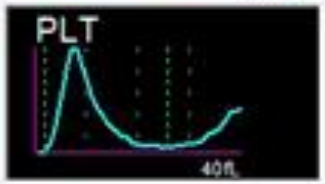
Main | Graph | Q-Flag | Service

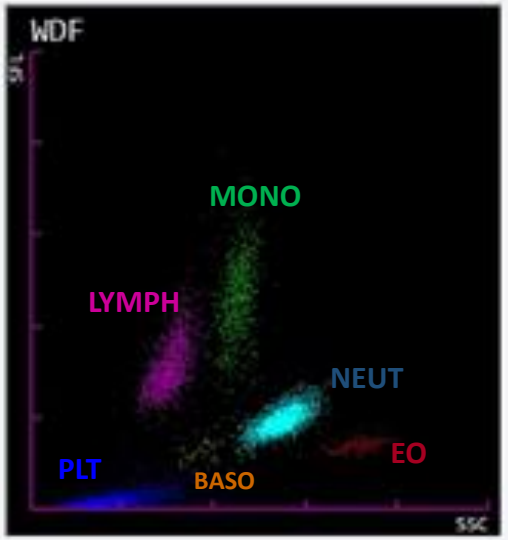
Item	Data		Unit
WBC	6.79		10^3/uL
RBC	6.23	+	10^6/uL
HGB	16.5		g/dL
HCT	49.1		%
MCV	78.8	-	fL
MCH	26.5		pg
MCHC	33.6		g/dL
PLT	267		10^3/uL
RDW-SD	48.8		fL
RDW-CV	18.0	+	%
PDW	12.1		fL
MPV	10.0		fL
P-LCR	25.7		%
PCT	0.27		%

Item	Data		Unit
NEUT#	4.15		10^3/uL
LYMPH#	1.53		10^3/uL
MONO#	0.86	+	10^3/uL
EO#	0.19		10^3/uL
BASO#	0.06		10^3/uL
NEUT%	61.1		%
LYMPH%	22.5		%
MONO%	12.7		%
EO%	2.8		%
BASO%	0.9		%
IG#	0.01		10^3/uL
IG%	0.1		%

Main | Graph | Q-Flag | Service







Cell counting in whole blood and isolated cell fractions

Total number of **PLT** and cells in **different populations of WBC**

Conclusions

- The purity of target cells fraction should be determined in every preparation for quality control
- For comparability of the results **O_2 fluxes should be corrected for contribution from contaminating cells** if contamination exceeds a certain threshold
- **Normalization of O_2 fluxes per cell count of target cells yields the most consistent results**

Contamination	*IO2*	CS	Protein
2.5 PLT/1 PBMC PBMC fraction	**5 %**	**10.5 %**	**7.5 %**
1.5 PBMC /1000 PLT PLT fraction	**5 %**	3 %	3.9 %

- **Find protocol with minimum contamination of PBMC fraction with PLT**

Protocols for isolation of PLT

1. Prevention of PLT activation:

Anticoagulants: K_2EDTA best yield and prevents activation of PLT
tested heparin, Citrate and Acid Citrate Dextrose
(Sjovall et al., Mitochondrion 2013)

Temperature – activation of PLT bellow 20 °C?

- storage of blood at 4°C for 1-3 h (EDTA + citrate, theophylline, adenosine, dipyridamole) – no sign of activation of PLT (MPV and MPC) *(Macey et al., Clinical Chemistry 2002)*

- storage of PLT at 4°C *(Bynum et al. Transfusion 2016)*

How to detect routinely PLT activation in blood sample, PLT fraction ?

PDW, MPV, PTC ?

Protocols for isolation of PLT

Centrifugation: **2. selection of PLT subpopulation**

- 300 *g* 15 min, RT -> PRP *(Sjovall et al., Mitochondrion 2013)*
  4600 *g* 5 min, RT -> PLT

  respiration: ce: own plasma
  pce: PBS+5 mM glucose, MiR05 *(Ehringer et al. J. Neurol. 2015)*

- 200 *g* 10 min, 25 °C, respiration ce: PRP *(Hroudova et al, Mitochondrion 2013)*
  pce: PRP+KH medium

- 500 *g* 15 min; **1500 *g* 8 min**, PGI2 *(Kramer et al. JOVE 2014),* washing and resuspension in PBS+PGI2

- **Lodz:** 3.2 % sodium citrate, 190 *g* 12 min + PGE1; **1000 *g* 12 min 37°C**, washing in Tyrode´s buffer, respiration in MiR05

- **Timisoara:** 500 *g* 10 min; **4600 *g* 5 min**, RT

3. Prevention of PLT activation during isolation procedure

- washing media
- time delay between isolation and respiration
- respiration media

? ? ?

Can PDW(fl) and MPV(fl) help to monitor PLT activation?

date	time	number	sample	ID	PLT	PDW(fL)	PDW/M	MPV(fL)	MPV/M
13/01/2017	10:00:35	1	blood	1	282	11.3		9.6	
13/01/2017	11:41:56	3	PBMC	1	39	13		12.2	
13/01/2017	11:46:41	5	PBMC	1	34	13.1		11.8	
13/01/2017	12:05:34	7	PLT	1	336	11		10.6	
13/01/2017	12:10:06	9	PLT	1	318	10.7	*	10.6	*
13/01/2017	10:02:08	2	blood	2	157	16.3		12	
13/01/2017	11:43:03	4	PBMC	2	25	19	*	14.2	*
13/01/2017	12:00:14	6	PBMC	2	24	22.3	*	14.5	*
13/01/2017	12:06:39	8	PLT	2	91	14.5	*	12.6	*
13/01/2017	12:11:20	10	PLT	2	92	15.6	*	12.8	*
07/02/2017	08:24:01	1	blood	9	166	16.1		12.2	
07/02/2017	09:42:18	3	PBMC	9	12	22	*	14.9	*
07/02/2017	09:43:22	4	PBMC	9	11	20.4	*	15.3	*
07/02/2017	09:44:32	5	sup PBMC	9	12	18.4		13.2	
07/02/2017	09:54:35	6	sup PLT	9	9	9.5		10.3	
07/02/2017	09:57:09	7	PLT	9	111	15.8	*	13	*
07/02/2017	09:58:20	8	PLT	9	106	17.1	*	13.1	*
07/02/2017	10:00:09	9	plasma	9	1	----		----	
07/02/2017	10:08:39	10	PBMC in cryo	9	6	21	*	15.1	*
07/02/2017	10:16:55	11	PLT in cryo	9	145	17	*	13.5	*
14/02/2017	09:32:46	1	blood	11	293	10.2		9	
14/02/2017	10:43:45	4	PBMC	11	15	10.6		10.9	
14/02/2017	10:45:40	5	PBMC	11	15	12.2		11.1	
14/02/2017	10:53:24	6	plasma	11	6	4.6		6.6	
14/02/2017	10:54:33	7	PLT	11	358	9.4	*	9.9	*
14/02/2017	10:55:37	8	PLT	11	328	10.1	*	10	*
14/02/2017	11:09:39	9	PBMC in cryo	11	13	11.8		11.7	
14/02/2017	11:16:52	10	PBMC in cryo	11	13	13.5		11.1	
14/02/2017	11:19:49	11	PLT in cryo	11	788	9.5		9.7	

Can PDW(fl) and MPV(fl) help to monitor PLT activation?

A

	PDW(fL)	MPV(fL)
median	**12.25**	**10.2**
mean	12.0	10.1
sd	1.13	0.65
n	9	9

B

	PDW(fL)	MPV(fL)
ID2	**16.3**	**12**
ID9	**16.1**	**12.2**

PDW(fl) and MPV(fl) in whole blood from healthy young men. Blood samples were counted on Sysmex XN-350 haematology analyser. **A)** Normal values from 9 blood samples. **B)** Values of PDW above 16 fl together with MPV above 12 fl indicate activation of platelets in blood sample that significantly affected respiration of PBMC and PLT – these samples were excluded for final evaluation.

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Univ.-Prof. Dr. Martin Burtscher, Verena Menz

SME: Sporttherapie Mag. Huber GmbH, Innsbruck, STH
Mag. Reinhard Huber

Stephanie Droescher, Verena Laner, Oroboros team

Thank you for your attention

Other isolation protocols in literature

Ficoll Paque Plus protocol – isolation of PBMC

2. Balanced salt solution. At least 20 ml for each sample to be processed. The balanced salt solution may be prepared from two stock solutions, A and B.

Solution A

		Conc. g/l
Anhydrous D-glucose	5.5×10^{-3} M (0.1%)	1.0
$CaCl_2 \cdot 2H_2O$	5.0×10^{-3} M	0.0074
$MgCl_2 \cdot 6H_2O$	9.8×10^{-4} M	0.1992
KCl	5.4×10^{-3} M	0.4026
TRIS	0.145 M	17.565

Dissolve in approximately 950 ml distilled water and add conc. HCl until pH is 7.6 before adjusting the volume to 1 l.

Solution B

		Conc. g/l
NaCl	0.14 M	8.19

To prepare the balanced salt solution, mix 1 volume of solution A with 9 volumes of solution B. Prepare the solution freshly each week. Other standard salt solutions may be used.

Ficoll Paque Plus protocol continues

- 3 mL of Ficoll Paque Plus + 4 mL diluted (1:1) blood in 10 mL tube
- 400 *g* 30-40 min, 18-20°C
- take layer, dilute with 3 volumes of the buffer
- centrifuge 60-100 g 10 min (2x)
- resuspend in appropriate medium

Typical results from our laboratories

Lymphocytes:	60 ± 20%	recovery of lymphocytes from the original blood sample
	95 ± 5%	of cells present in the lymphocyte fraction are mononuclear leukocytes
	>90%	viability (measured by trypan blue exclusion)
Other cells:	3 ± 2%	granulocytes
	5 ± 2%	erythrocytes
	<0.5%	of the total platelet content of the original blood sample

Ficoll-Hypaque instruction: Isolation of mononuclear cells

- 10 mL the blood/PBS mixture is slowly layered over 5 mL of Ficoll-Hypaque solution
- Centrifuge 20 min at 900 *g*, 25 °C, with no brake (decel -1).
- Transfer the mononuclear cells layer adding excess PBS/2 mM EDTA (3 times the volume), centrifuge 3-5 min at 400 *g*, 22-25 °C.
- Resuspend cells in PBS/2 mM EDTA, and repeat the wash.
- **To remove the extra platelets layer the cell suspension (0.5 mL) over 3 mL FBS**, centrifuge 10 min at 300 *g*, 22-25 °C.
- Resuspend cells in PBS.

jove Journal of Visualized Experiments

Video Article

Bioenergetics and the Oxidative Burst: Protocols for the Isolation and Evaluation of Human Leukocytes and Platelets

Philip A. Kramer*[1], Balu K. Chacko*[1], Saranya Ravi[1], Michelle S. Johnson[1], Tanecia Mitchell[1], Victor M. Darley-Usmar*[1]

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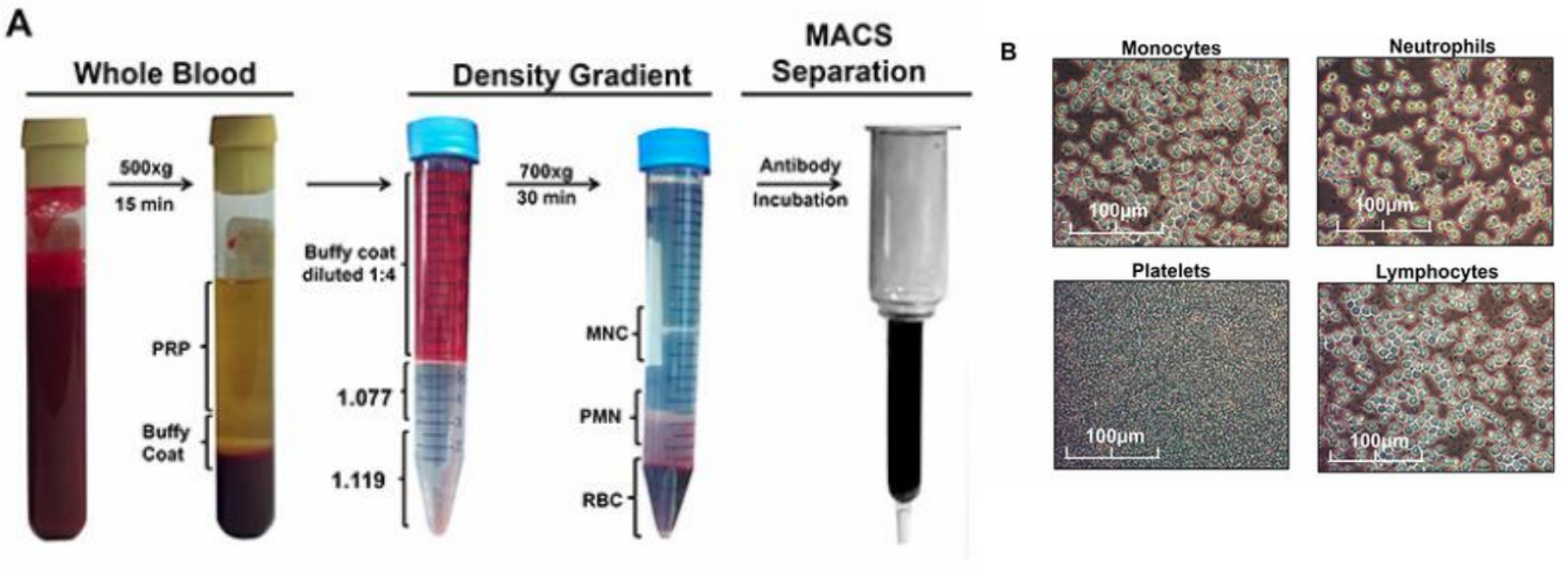

RPMI 1640 without glutamine and phenol red

Prague method: isolation of lymphocytes

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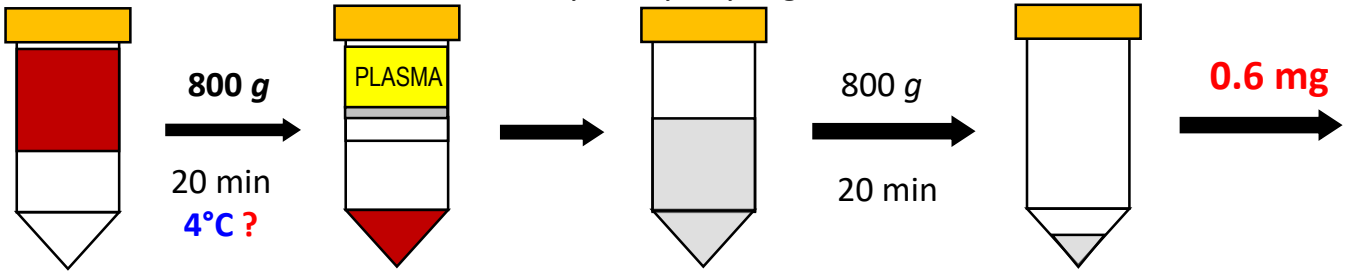
Noninvasive diagnostics of mitochondrial disorders in isolated lymphocytes with high resolution respirometry

Petr Pecina [a], Hana Houšťková [b], Tomáš Mráček [a], Alena Pecinová [a], Hana Nůsková [a], Markéta Tesařová [c], Hana Hansíková [c], Jan Janota [d], Jiří Zeman [c], Josef Houštěk [a,*]

EDTA tubes
no dilution of blood



Ficoll Paque
PLUS

20 min **on ice**
? effect

PBS+protease inhibitors ?
sediment

30°C
KCl medium ?