METABOLOMICS • SAMPLE PREPARATION • BLOOD COMPONENTS

Evaluation of Red Blood Cell Depletion in Blood Fractionation Workflows

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Introduction

On average, 50% of blood is comprised of the cellular material. Of the cellular component, 99% is the red blood cells (erythrocytes, RBCs). RBCs will dominate cellular 'omics signals without depletion.

In our previous study, we explored the utility of RBC lysis as a mechanism for the enrichment of white blood cells (leukocytes, WBCs). However, we noted that initial collection tube choice potentially impacted lysis efficacy.

In this study, we demonstrate that **RBC lysis is affected by the choice of the initial collection vacutainer tube**. Here we discuss how the initial choice of tube anticoagulant affects the lysis efficacy for several commonly used clinical blood collection tubes.

Plasma and RBC pellet fractions were collected by centrifuging whole blood at 1300 x g for 10 min. The supernatant (plasma) was transferred to a new tube, and the remaining RBC pellet was washed twice with equal volume PBS.

Samples were carried through into FACS analysis within 2 hours of lysis following labeling of CD45 (leukocytes) and CD235a (erythrocytes) surface markers. Fractions were scored based on residual percent RBC, reflecting suboptimal lysis.

In parallel, metabolites were extracted using 80:20 methanol:water, separated by HILIC and C18 chromatography, and assessed on an Agilent 6545 Q-TOF in both positive and negative ionization modes. Agilent Profinder and Mass Profiler Professional were used for untargeted feature extraction and statistical analysis.

Highlights

- Red blood cells dominate the cellular material in blood while other cellular components, such as the leukocytes, are often the target of interest.
- **Density centrifugation** and **RBC lysis** are two common methods to deplete red blood cells.
- **Density centrifugation** separates granulocytes and RBCs from the less dense peripheral blood mononucleocytes (PBMCs, including B- and T-cells) while **RBC lysis** relies on the Band 3 anion transport protein present in erythrocytes to lead to osmolarity induced cellular disruption.
- We have identified metabolomic markers of RBCs vs granulocyte rich and poor WBCs. We have confirmed these results via Fluorescence-activated cell sorting (FACS).
- **Here we report that Erythrocyte lysis depletion via ammonium chloride potassium (ACK) based lysis is critically dependent on collection tube formulation.**

Methods

Twelve blood draws were performed over three sample collection events. For each blood draw, approximately 24 mL of blood were collected into 5 different tubes: K2-EDTA, K3-EDTA, Na-Citrate, ACD and Na-Heparin. From each collection tube, 1 mL of whole blood was carried through for lysis by addition into 9 mL ACK lysis buffer (0.15 M ammonium chloride, 10 mM potassium bicarbonate, 0.1 mM EDTA) for 20 minutes at 4 °C. The solution is isoosmotic, however, the Band 3 Anion transporter present in RBCs leads to osmotic dysregulation and membrane disruption. The resultant WBC pellet was 23.6 +/- 9.1 mg.

Results

Red Blood Cell Lysis is dependant on initial collection tube with K2-EDTA being found to be the most efficacious with ~1% RBCs (comparable to our CPT results). K3-EDTA was the next most efficacious (3% RBCs) while Na-Citrate, ACD and Na-Heparin tubes had very extensive residual RBC contamination (30-50%). While many protocols have shown results that are independent of blood collection tube modality[1,2], these findings firmly reiterate the importance that validated protocols are often "fit-for-purpose" and **must be** revalidated when used outside of the original intended use.

[1] Chen, H., Schürch, C.M., Noble, K. et al. Functional comparison of PBMCs isolated by Cell Preparation Tubes (CPT) vs. Lymphoprep Tubes. BMC Immunol 21, 15 (2020). https://doi.org/10.1186/s12865-020-00345-0

[2] Gautam A, Donohue D, Hoke A, Miller SA, Srinivasan S, Sowe B, Detwiler L, Lynch J, Levangie M, Hammamieh R, Jett M. Investigating gene expression profiles of whole blood and peripheral blood mononuclear cells using multiple collection and processing methods. PLoS One. 2019 Dec 6;14(12):e0225137. doi: 10.1371/journal.pone.0225137. PMID: 31809517; PMCID: PMC6897427.

Additional findings:

In comparing density vs lysis preparations, notable is the presence of the granulocyte population in the lysis workflows that is absent in density centrifugation workflows.

LC-MS analysis of the extracted cell pellets yielded significant features that allow for characterization of either unfractionated or RBC depleted blood. Of the significant features, 161 were identified via our internal RT/MZ library. These were further curated to a list of 25 markers for RBC contamination whose normalized intensities mirror the FACS results showing the largest signal reduction in the K2-EDTA group.

Acknowledgements

Funding in part for these efforts was provided from Uniformed Services University of the Health Sciences (USUHS) award from the Defense Health Program to the Murtha Cancer Center Research Program (HU00011820032, JE Katz/JSH Lee) administered by the Henry M. Jackson Foundation for the Advancement of Military Medicine. Disclaimer: The contents of this publication are the sole responsibility of the authors and do not necessarily reflect the views, opinions, or policies of the USUHS, the Henry M. Jackson Foundation for the Advancement of Military Medicine, Inc., the Department of Defense (DoD), the Departments of the Army, Navy, or Air Force. Mention of trade names, commercial products, or organization does not imply endorsement by the U.S. Government. We further thank the generous effort and intellectual contribution from the Windber Research Institute without which this project would not have been possible.

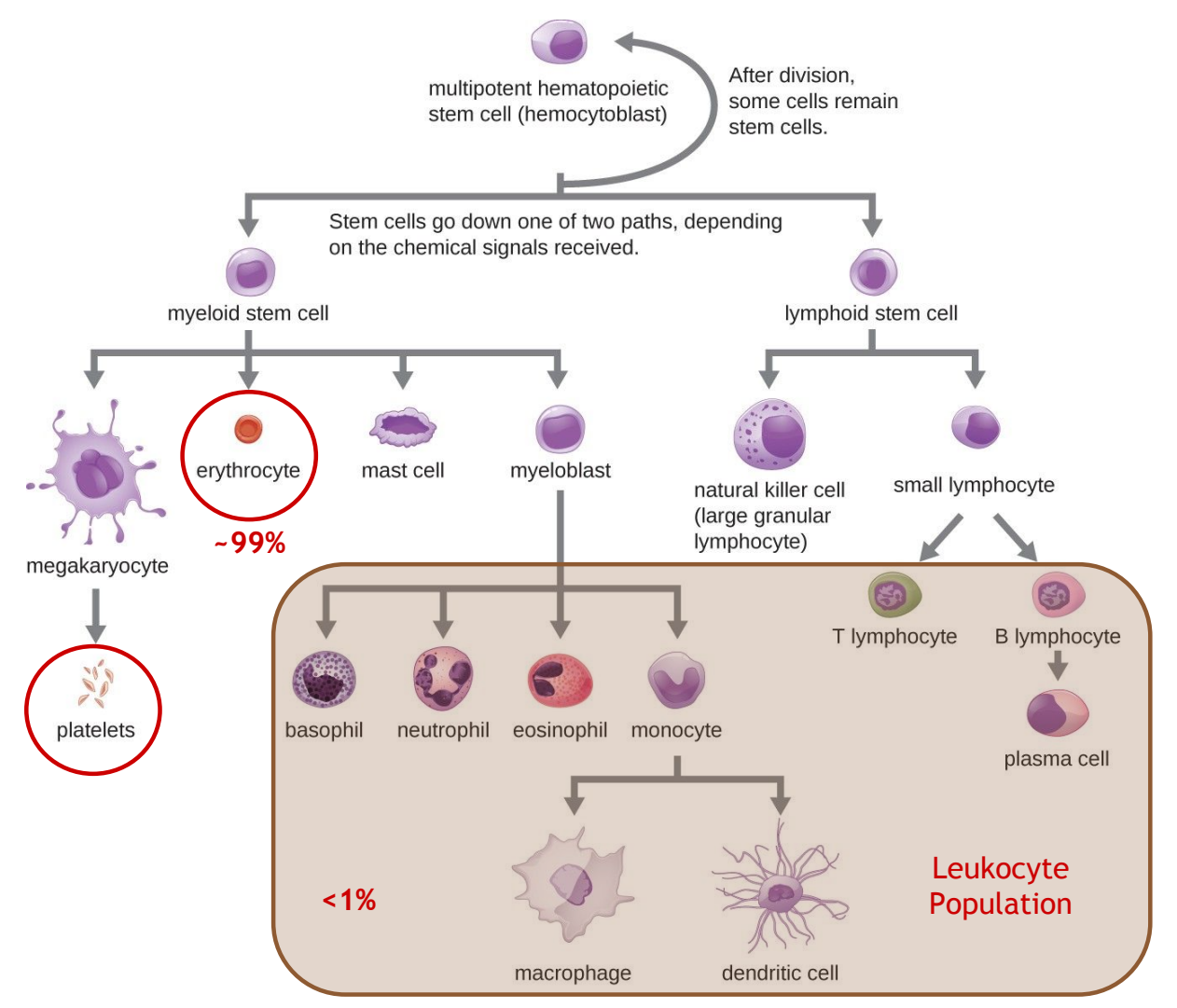
Conflict of Interest

The authors declare no competing financial interest.

Cellular Composition of Blood

Red Blood Cells Make Up ~99% of Cellular Material in Blood

Signals from "reactive" white blood cells are dominated by signals from RBCs

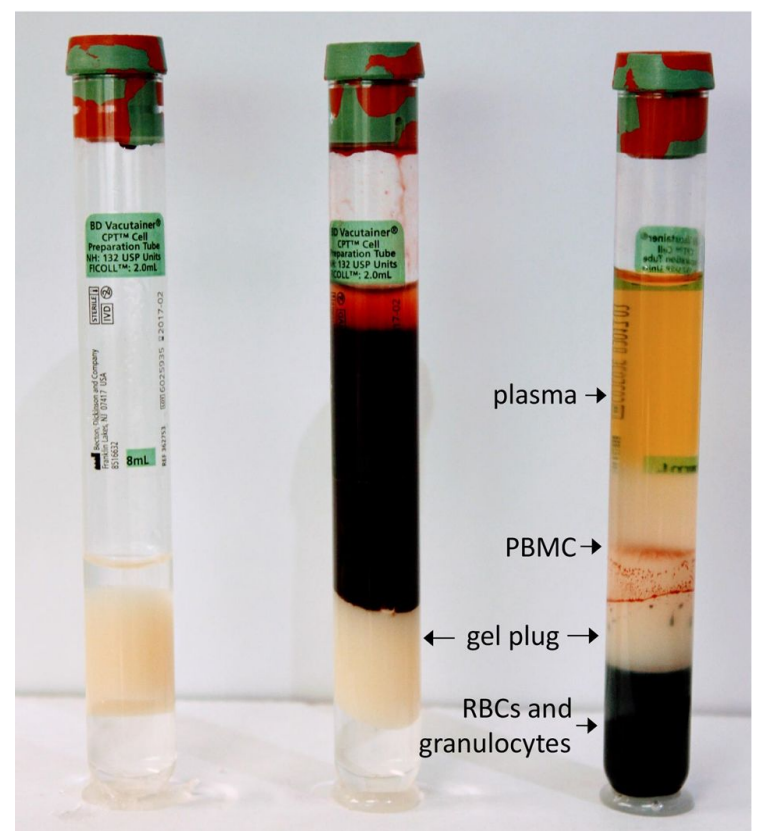


adapted from: https://courses.lumenlearning.com/suny-microbiology/chapter/cellular-defenses/

Figure 1: The cellular composition of blood. Blood contains red blood cells (RBCs, erythrocytes), white blood cells (WBCs, leukocytes), and platelets (thrombocytes). The leukocyte group encompasses many specialized cell sub-types that are often of interest to study due to their unique functions and the information they carry about the metabolic and health state of the individual they belong to: leukocytes are considered the reactive component of the immune system. However, the volume of cellular material in blood is made of ~99% erythrocytes, which dominate measurement signals and necessitate selective reduction strategies in order to isolate the cell population of interest.

Isolating White Blood Cells via Erythrocyte Depletion: Two Accepted Methods

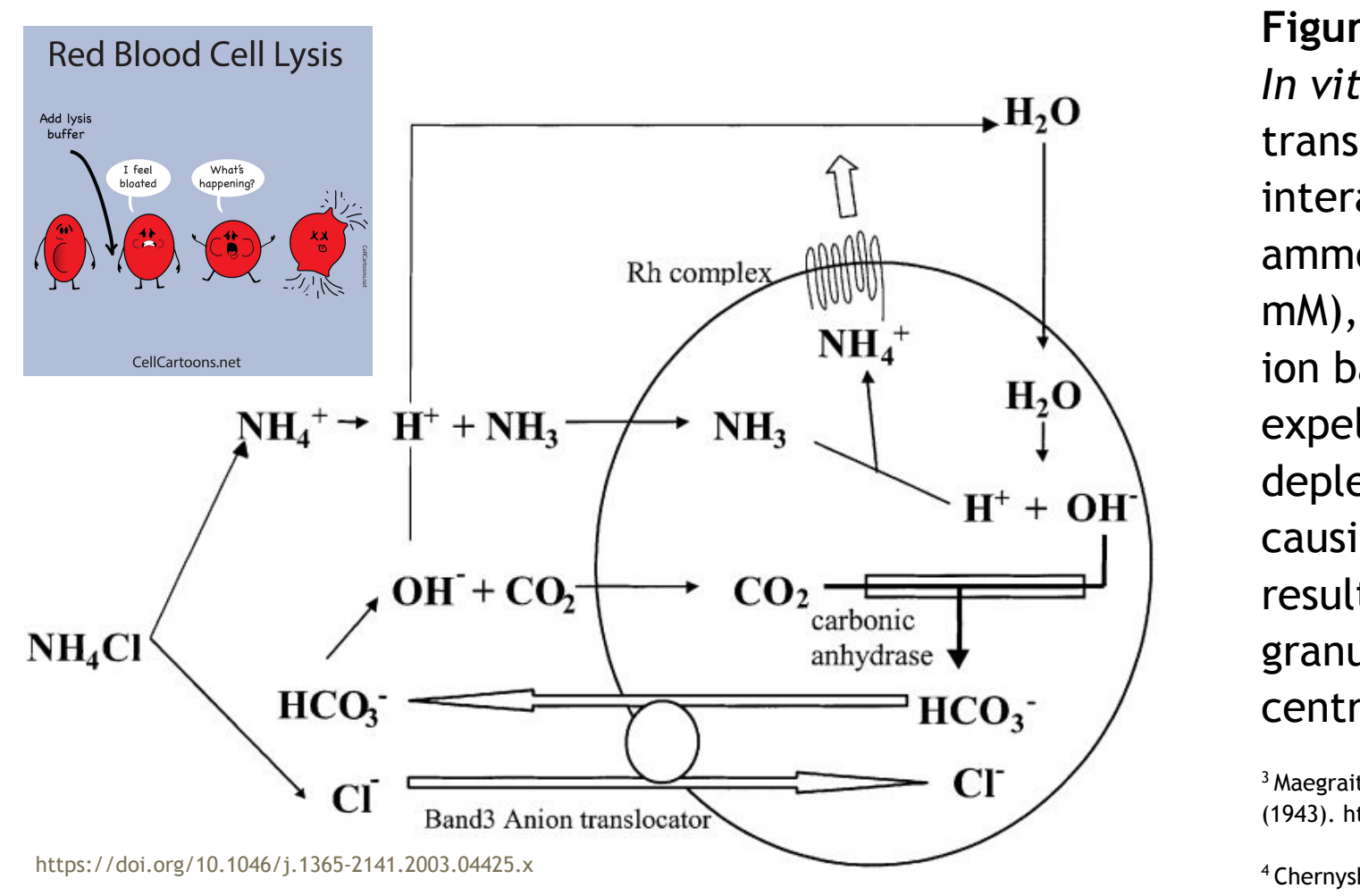
***Density Gradient Centrifugation** allows separation of cellular components based on differing cellular densities.*



Puleo, *et al*. 2017. DOI 10.21769/BioProtoc.2103.

Figure 2: BD CPT Density gradient centrifuge tubes. BD CPT cat no. 362753 are pre-filled with anticoagulant (sodium heparin), a liquid density medium, and an inert gel barrier. Centrifugation in these tubes at the right temperature and RCF results in layers in which RBCs and granulocytes separate from the remaining WBCs and plasma. The resulting WBC portion is the group of leukocytes classified as peripheral blood mononuclear cells (PBMCs).

***Red Blood Cell Lysis** uses ammonium chloride buffer to selectively lyse red blood cells without disturbing white blood cells.*



https://doi.org/10.1046/j.1365-2141.2003.04425.x

Figure 3: RBC Lysis Mechanism
In vitro RBC lysis is facilitated by Band 3, an anion transport protein in RBCs. Lysis is triggered when RBCs interact with an isotonic solution containing ammonium chloride (0.15M), potassium bicarbonate(10 mM), and EDTA (0.1 mM). The buffer disrupts the RBCs' ion balance, inducing an influx of chloride ions and expelling bicarbonate ions. This causes CO_2 and H_2O depletion, leading to water entering the RBCs and causing lysis. The process leaves leukocytes intact, resulting in a pure leukocyte suspension, including granulocytes often lost in density gradient centrifugation[3,4].

[3] Maegraith, B., Findlayyl, G. & Martin, N. Mechanism of Lysis of Red Blood Cells. Nature 151, 252–253 (1943). https://doi.org/10.1038/151252b0

[4] Chernyshev AV, Tarasov PA, Semianov KA, Nekrasov VM, Hoekstra AG, Maltsev VP. Erythrocyte lysis in isotonic solution of ammonium chloride: theoretical modeling and experimental verification. J Theor Biol. 2008 Mar 7;251(1):93-107. doi: 10.1016/j.jtbi.2007.10.016. PMID: 18083194.

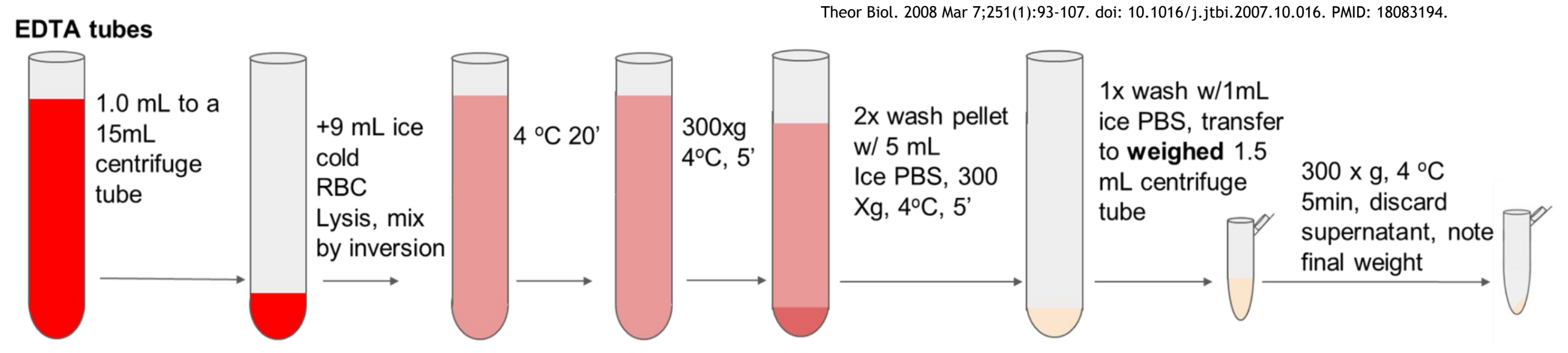


Figure 4: Experimental workflow for red blood cell lysis. Blood is mixed with lysis buffer (ammonium chloride potassium (ACK) solution, BioLegend catalog #420301) and incubated for 20 minutes. The remaining leukocytes are washed twice with PBS to remove RBC debris.

RBC Depletion via ACK Lysis Performs Comparably to Density Separation with CPT Tubes, but is Critically Dependent on Anticoagulant Tube Choice

WBC Isolation Efficacy: Metabolomics

Metabolic markers of RBCs show comparable efficacy in CPT vs K2-EDTA Lysis

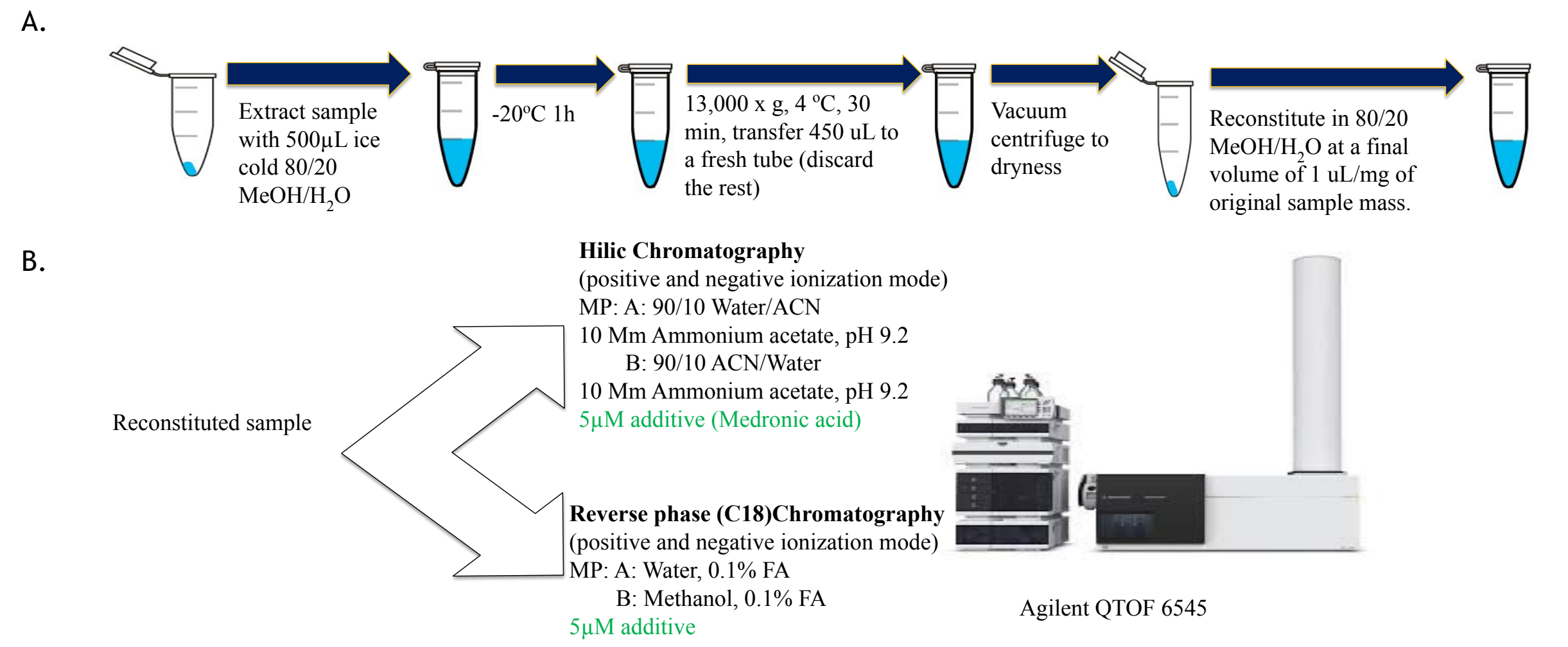


Figure 5: Metabolomics workflow for A) metabolite extraction and B) LC-MS analysis.

Table 1: Metabolites enriched in RBC preparation

Compound	RBC peak area	RBC/CPT Intentity Ratio	RBC/Lysis Intentity Ratio	RBC/Plasma Intentity Ratio	RBC/Plasma from literature[5]
5-Methyl Ergothioneine	5.98E+05	37.4	46	66	69.6
Acetyl-Carnitine	1.51E+05	**11.3**	**5.1**	2.7	2.6
Adenine	5.19E+05	21.5	22.6	2.8	2.4
Adenosine 5'-Monophosphate	1.19E+05	5.1	3.2	39.2	40.3
Aspartate	4.37E+05	7.7	8.1	44.2	41.1
Carnosine	9.45E+04	4.6	2.7	9	10.8
Citramalate	3.04E+05	9	5.1	2.2	2.4
Creatine	1.11E+07	874	460.6	0.9	0.9
Dimethyl-Proline	1.16E+05	4.3	4.6	2.1	2.6
Ergothioneine	1.37E+06	42.9	26.3	87.1	77.8
Fructose-6-Phosphate	6.04E+04	4.4	3.8	26.1	28.1
GDP	5.43E+04	3.3	3	19.6	24.1
GDP-Glucose	4.38E+04	2.5	1.8	36.7	32
Gluconate	4.81E+05	21.7	21.2	14.8	11.8
Glutamate	1.13E+05	2.7	2.3	4.1	4.9
Glutathione Disulfide	1.28E+05	3.4	2.5	**853.3**	**1603.9**
Inosine 5'-Phosphate	3.55E+05	10.3	9	**95.5**	**132.2**
L-2-Phosphoglyceric Acid	2.36E+04	0.3	0.3	94.4	93.5
Malate	5.24E+05	6.5	6.8	4.2	3.6
N-Acetyl-D-Glucosamine	1.39E+05	1.6	1.7	**8.2**	**10.3**
N-Acetyl-Glutamate	1.15E+05	2.1	2.2	2.4	2.1
NADP	1.14E+05	2.3	2.6	ND in plasma	ND in plasma
Nicotinamide	1.55E+05	12.8	14.8	ND in plasma	ND in plasma
Ophthalmic Acid	6.23E+04	1.9	1.5	ND in plasma	914.9
Phosphocreatine	1.43E+05	1.9	1.7	**2**	**2.8**
Propionyl Carnitine	1.32E+05	1.4	1.3	6	7.4
S-(5'-Adenosyl)-L-Methionine	2.97E+05	4.4	4.6	13.6	16.3
Succinate	1.06E+05	1.2	1.3	2.6	2.4
Trimethyl-Lysine	1.02E+05	2.1	2.3	5.2	6.2
Trimethyl-Phenylalanine	1.08E+05	2	2	4.3	5.3
Trimethyl-Tyrosiine	1.35E+05	2	2.1	38.5	37.5
UDP-Glucose	2.89E+05	4	3.8	205.4	227.2
UDP-Glucuronate	1.10E+05	2	2	54.4	47.4
Urate	8.12E+05	**21.9**	**9.4**	7.1	6.5
Uridine 5'-Diphosphate	2.39E+05	2.7	2.6	41.5	46.2
Uridine 5'-Monophosphate	4.21E+05	7.1	7.1	47.1	46.8
Uridine 5'-Triphosphate	3.06E+05	3.3	3.3	1.7	1.6

[5] Chaleckis R, Murakami I, Takada J, Kondoh H, Yanagida M. Individual variability in human blood metabolites identifies age-related differences. Proc Natl Acad Sci U S A. 2016 Apr 19;113(16):4252-9.

Leukocytes were isolated using either density gradient centrifugation from blood collected directly into CPT tubes or ammonium chloride potassium (ACK) lysis from blood collected into K2-EDTA vacutainer tubes. Leukocyte, plasma, and RBCs were extracted using the protocol in Figure 5. MS features were identified against our in house library (retention time & m/z). Intensity value ratios between red blood cell extracts and leukocyte extracts prepared from density gradient with CPT tubes (column 3) or RBC lysis (column 4) are shown. Values for metabolites with a difference factor between CPT and RBC lysis of >1.5 are shown in orange, and those with a difference factor >2.0 between CPT and lysis are shown in red. Columns 5 & 6 show a similar analysis using the ratio of compound intensities from our measured RBC extracts and plasma versus values reported in literature. Values with a percent change from literature greater than 20% are shown in blue.

The metabolites identified in Table 1 act as potential markers for the presence (or contamination) of erythrocytes in processed blood samples. RBC lysis using an ammonium-chloride-potassium solution shows comparable results to density gradient centrifugation with CPT tubes. Some of the observed metabolite differences between CPT and lysis have been previously reported (such as for Urate[6] and Acylcarnitine[7]).

[6] Shaw OM, Steiger S, Liu X, Hamilton JA, Harper JL. Brief report: Granulocyte-macrophage colony-stimulating factor drives monosodium urate monohydrate crystal-induced inflammatory macrophage differentiation and NLRP3 inflammasome up-regulation in an in vivo mouse model. Arthritis Rheumatol. 2014 Sep;66(9):2423-8. doi: 10.1002/art.38730. PMID: 24910235.

[7] Katrib K, Adlouni HA, Férard G. Presence of nonesterified and acylcarnitine in human polymorphonuclear leukocytes and mononuclear cells. Clin Chem. 1987 Apr;33(4):533-5. PMID: 3829385.

WBC Isolation Efficacy: FACS (Fluorescence-Activated Cell Sorting) Analysis

Cell population quantification with FACS shows highest RBC depletion with K2-EDTA anticoagulant

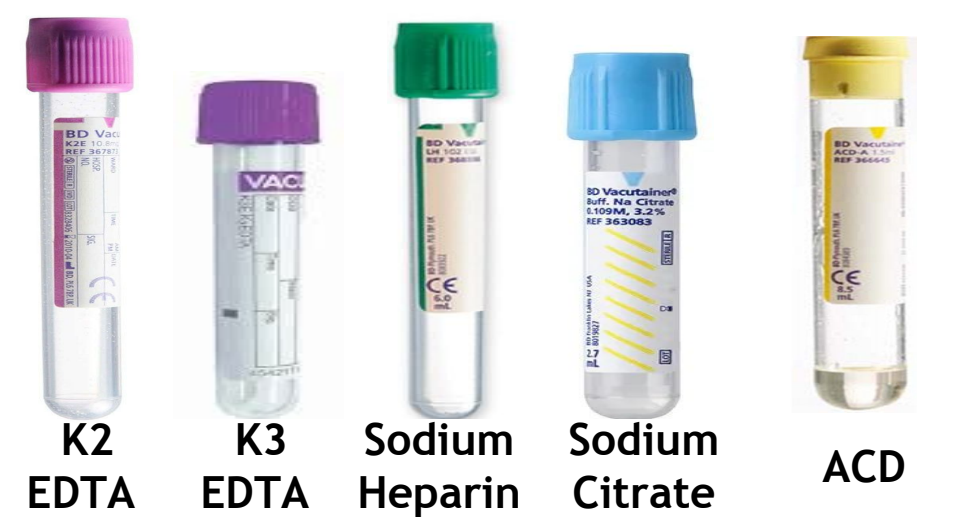


Figure 6. Exemplar anticoagulant tubes.

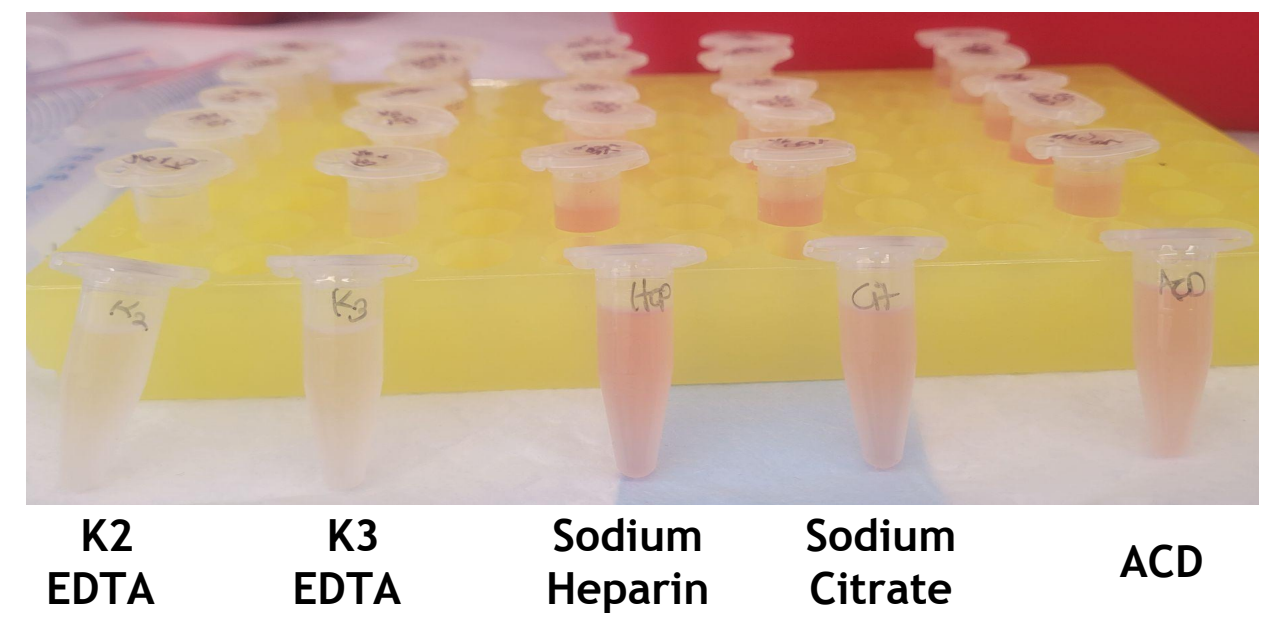


Figure 7. Incomplete lysis is visually evident during RBC lysis wash step.

We selected five of the most common blood collection tubes to compare with the RBC lysis protocol.

Efficacy of RBC lysis using ACK solution is visually apparent. **Figure 7** shows representative samples from each anticoagulant tube after conclusion of the lysis step, during the first wash step. Samples processed from vacutainer tubes containing sodium heparin, sodium citrate, and anticoagulant citrate dextrose (ACD) are noticeably pink in contrast to those from K2-EDTA and K3-EDTA tubes.

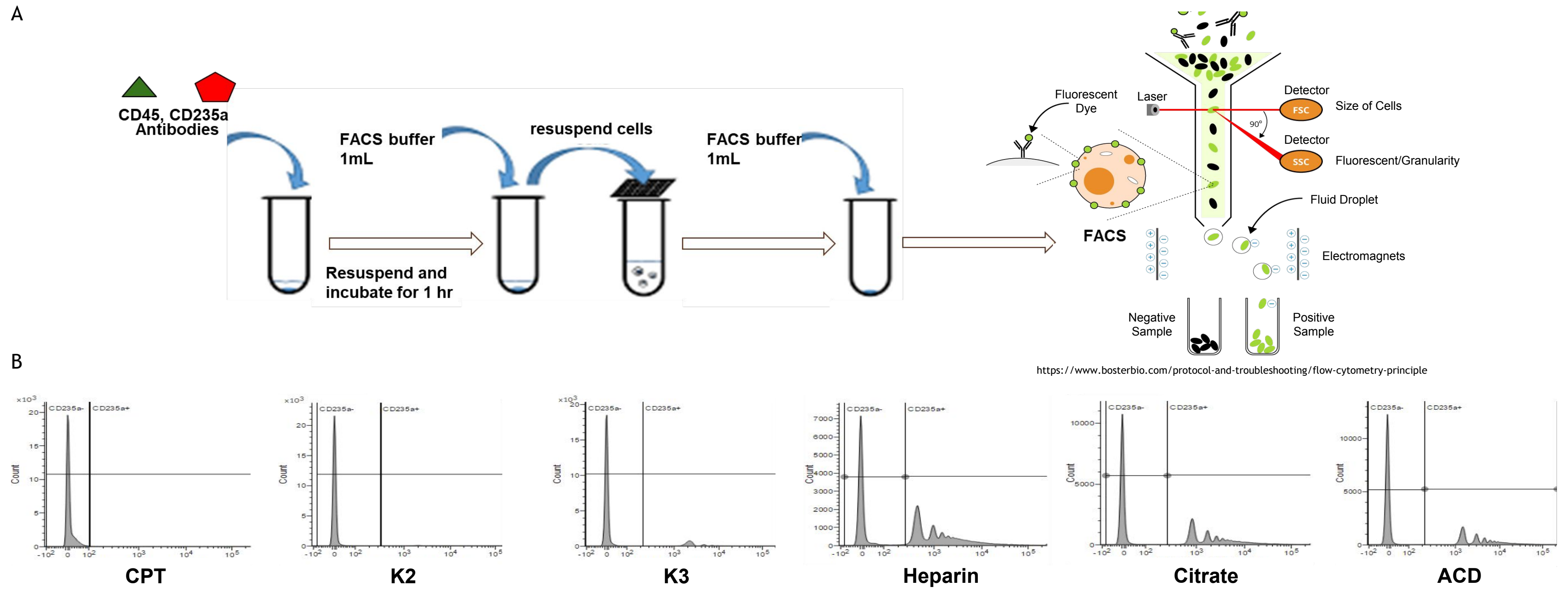


Figure 8. A) FACS workflow. Blood cells were stained with CD45 and 235a markers for FACS analysis. **B) RBC contamination.** Histograms with fluorescence intensity for CD235a+ in PBMCs isolated from blood collected in different anticoagulant blood collection tubes.

We chose to use FACS to quantify the populations of erythrocytes and leukocytes to complement metabolomics results and further investigate the impact of blood collection tube on the efficacy of RBC lysis. Leukocyte suspensions isolated from whole blood collected into five types of anticoagulant tubes were characterized after depletion of RBCs using ACK lysis. CD235a+ labeling indicates the presence of contaminating RBCs that were not lysed and removed during RBC lysis, whereas CD45+ labeling verifies various populations of leukocytes: granulocytes, monocytes, and lymphocytes.

Table 2: Summary of cell population in different blood collection tubes (n=6)

Cell population	CPT*	K2	K3	Heparin	Citrate	ACD	RBC
% WBC (CD45+)	68.5±0.3	94.5±1.3	91.6±4.1	47.2±10.2	60.6±9.1	62.1±10	NA
% Granulocytes	**1.4±1.1**	72.1±6.3	66.8±7.1	35.5±6.5	37.1±3.4	39.8±5.3	NA
% Monocytes	**27.7±0.7**	3.9±1.5	3.8±1.4	2.5±0.2	2.7±.3	3.1±0.2	NA
% Lymphocytes	**38.0±0.1**	12.4±4.8	13.7±3.1	10.9±2.8	10.1±2.4	11.6±3.1	NA
% RBC (CD235a+)	0.5±0.5	1.1±0.5	3.1±0.5	54.7±3.1	37.5±5.3	34.0±1.4	94.5±2.7

* CPT values collected from a separate experimental batch than other table values

FACS results confirm the visual observations (Fig. 7) that using sodium heparin, sodium citrate, or ACD anticoagulant tubes for initial blood collection results in reduced efficacy of the RBC lysis step. Table 2 and Fig. 9 show that all non-EDTA anticoagulants tested produce a leukocyte suspension that is highly contaminated with RBCs (30%+). While RBC contamination remains much lower in the K3-EDTA condition (3.1%), **surprisingly only** K2-EDTA remains similar to density gradient centrifugation with CPT tubes (1.1±0.5% and 0.5±0.5% respectively).

Table 2 further quantifies the different leukocyte populations that are produced by the two RBC-depletion methods. Granulocytes are highly depleted in the CPT condition (~1%) compared with the lysis conditions (>35%).

The interesting result between K2- and K3-EDTA tubes poses the question of how the choice of anticoagulant used in blood collection impacts the mechanism for RBC lysis. Since the primary difference between K2- and K3-EDTA is an increase in potassium ions, we hypothesize that the 50% increase of [K^+] pushes the ammonium/chloride/bicarbonate equilibrium in the undesired direction, slowing the osmotic forces driving the mechanism and resulting in incomplete RBC lysis. Ionic sodium may be playing a similar role in the heparin, citrate, and ACD conditions in addition to potential osmotic balancing.

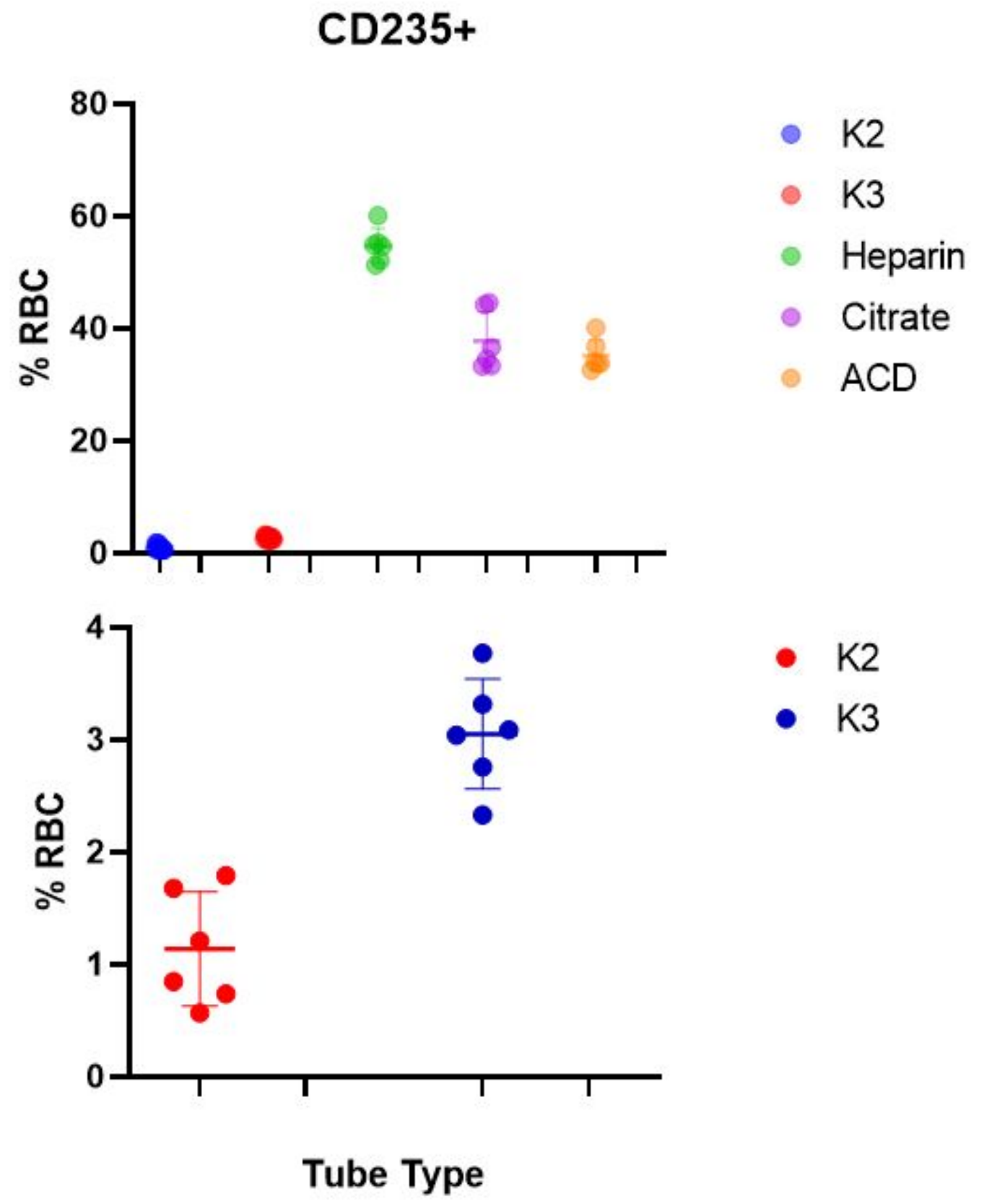


Figure 9. K2-EDTA tubes produce better lysis than K3-EDTA tubes. Residual red blood cell presence indicated by CD235+ labeling using FACS analysis on all tested anticoagulants (above) and scaled y-axis showing just K2- and K3-EDTA (below). Average values for each anticoagulant are shown in the "% RBC (CD235+)" row in Table 2.