MILITARY EXPOSURES AND SUBSEQUENT LONG-TERM OUTCOMES

Evaluation of Red Blood Cell Depletion in Blood Fractionation Workflows Towards a Leukocyte Based Exposure Assay

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Introduction

Metabolomics seeks to classify cancer and assess health state through small molecule measurements. White blood cells (WBCs, leukocytes, PBMCs) offer a "molecular history" of exposure, allowing potential insights into long-term health risks. Effective study of WBCs requires robust RBC depletion methods due to red blood cell (RBCs, erythrocytes) dominance (~99%) in metabolomics signals in blood (**Figure 1**).

RBC lysis with ammonium chloride potassium buffer (ACK, **Figures 3 & 4**) minimally impacts WBCs and offers a simple, cost-effective solution to isolate WBCs that is adaptable, *especially in remote sampling situations*.

In this study, we explore the **preanalytic variable of initial blood collection anticoagulant choice on RBC lysis efficacy** and compare RBC lysis to isopycnic centrifugation using CPT tubes (**Figure 2**) to isolate peripheral blood mononucleocytes (PBMCs). Additionally, we investigate the metabolic signatures of RBC contamination of WBCs. The findings inform future guidelines for rapid WBC/PBMC preparation, enhancing efforts to *assess health and exposure impacts*.

Highlights

- Effective study of WBCs requires robust RBC depletion methods due to red blood cell (RBC) dominance (~99%) in metabolomics signals in blood.
- Comparative effectiveness of various anticoagulant tubes (K2-/K3-EDTA, sodium heparin, sodium citrate, anticoagulant citrate dextrose (ACD)) in the RBC lysis process for WBC isolation is explored.
- We identified potential metabolomic markers distinguishing RBCs from granulocyte-rich and granulocyte-poor WBCs. These findings were validated using Flow Cytometry (FC), High-Content Imaging analysis, and Metabolomics analysis.
- We report that erythrocyte depletion via ammonium chloride potassium (ACK) lysis is critically dependent on formulation of the collection tube, significantly impacting data quality.

Results

RBC Lysis is dependant on initial collection tube, with K2-EDTA and K3-EDTA being found to be the most efficacious.
Na-citrate, ACD, and Na-heparin increased RBC contamination (>5%). 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.

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 RBC depleted blood. Of the significant features, several were further curated to a list for RBC contamination (**Table 1**) whose normalized intensities mirror the FACS results, showing the largest signal reduction in the EDTA anticoagulant groups (**Figure 13**).

[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.

Materials & Methods

Three donors blood (~36 mL of blood each) were collected into 5 different anticoagulant collection tubes (K2-EDTA, K3-EDTA, sodium citrate, sodium heparin, anticoagulant citrate dextrose (ACD)) and 2 density gradient CPT tubes (CPT + sodium citrate and CPT + sodium heparin) (**Figure 5**). All tubes were BD Vacutainer brand. From each collection tube, RBC lysis was performed with 1 mL of whole blood in 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 resultant WBC pellets were 31.7 +/- 8.1 (2xSE) mg.

Plasma and RBC pellet fractions were collected by centrifugation of whole blood (1300 x g, 10 min). The supernatant (plasma) was transferred to a new tube, and the remaining RBC pellets were washed twice with equal volume PBS (**Figure 4**).

To identify cell populations using imaging and flow cytometry analysis (FC), samples were diluted in 500 uL buffer (PBS + EDTA), of which 100 uL was used (approximately 1 x 10^6 cells each). For the viability staining, equal sample volume of 1:100 diluted Zombie NIR was used (20 min, RT, dark), washed off with FACS buffer 10x volume), and pelleted. Samples were then labeled with CD45 (leukocyte marker) and CD235a for (RBC marker) (40 min, RT, dark) and carried through into FACS analysis (**Figure 13**) and High Content Imaging analysis (**Figure 12**). 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 chromatographies, and assessed on an Agilent 6545 Q-TOF in both positive and negative ionization modes (**Figure 7**). Agilent Profinder and Mass Profiler Professional were used for untargeted feature extraction and statistical analysis.

Conflict of Interest

The authors declare no competing financial interest.

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.

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Cellular Composition of Blood

Red Blood Cells Make Up ~99% of Cellular Composition in Blood
Signals from WBCs are obscured by overwhelming signals from RBCs

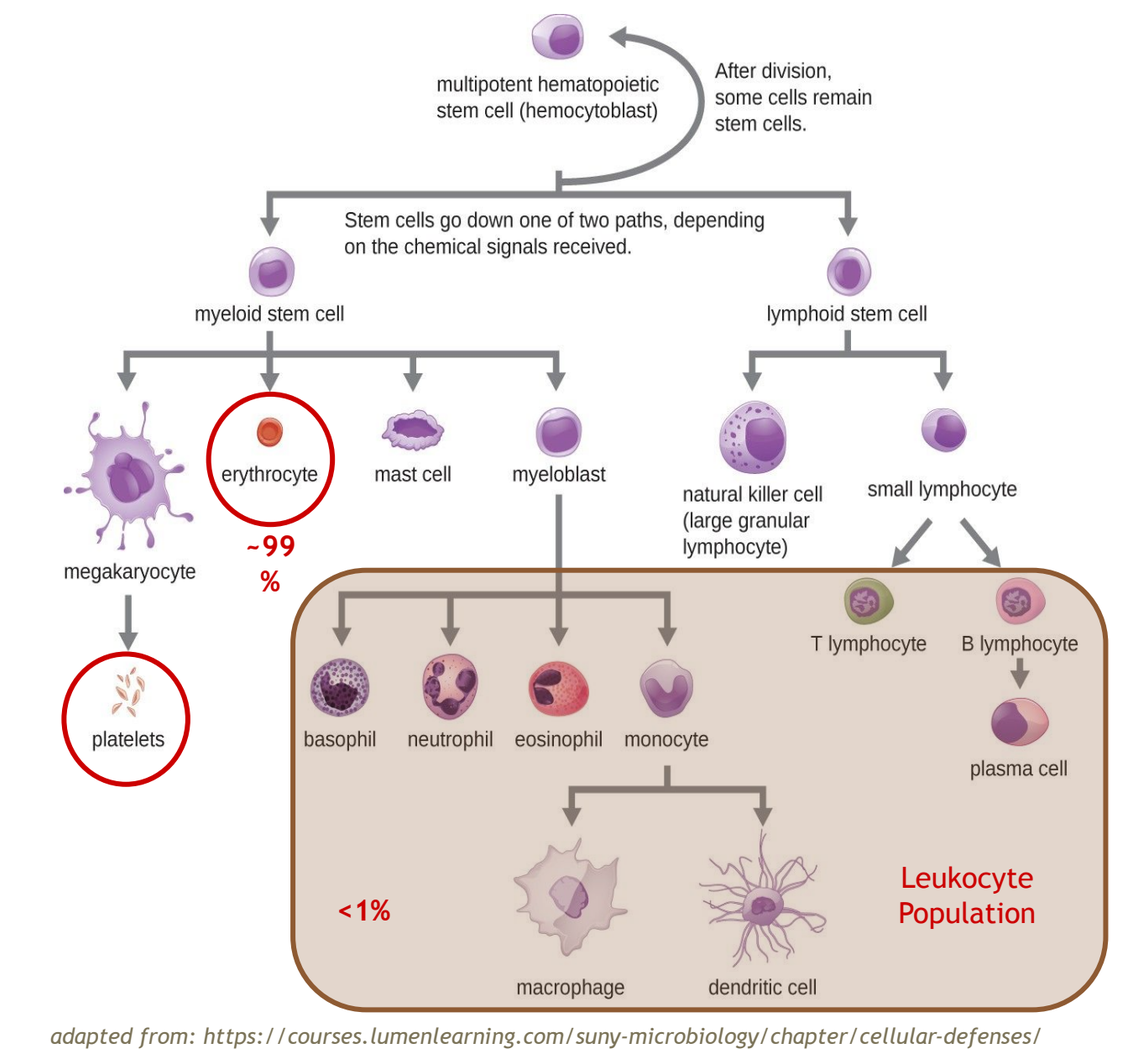


Figure 1: The cellular composition of blood
Blood is composed of red blood cells (RBCs, erythrocytes), white blood cells (WBCs, leukocytes), and platelets (thrombocytes). Among these, the WBC group includes specialized cells that are of interest due to their distinct functions and the insights they provide about health, exposure, and the immune system. However, the RBCs that make up ~99% of blood's cellular content dominate measurement signals and require prior selective depletion to isolate the cell population of interest (WBCs).

Methods of Red Blood Cell Depletion

Density Gradient Centrifugation

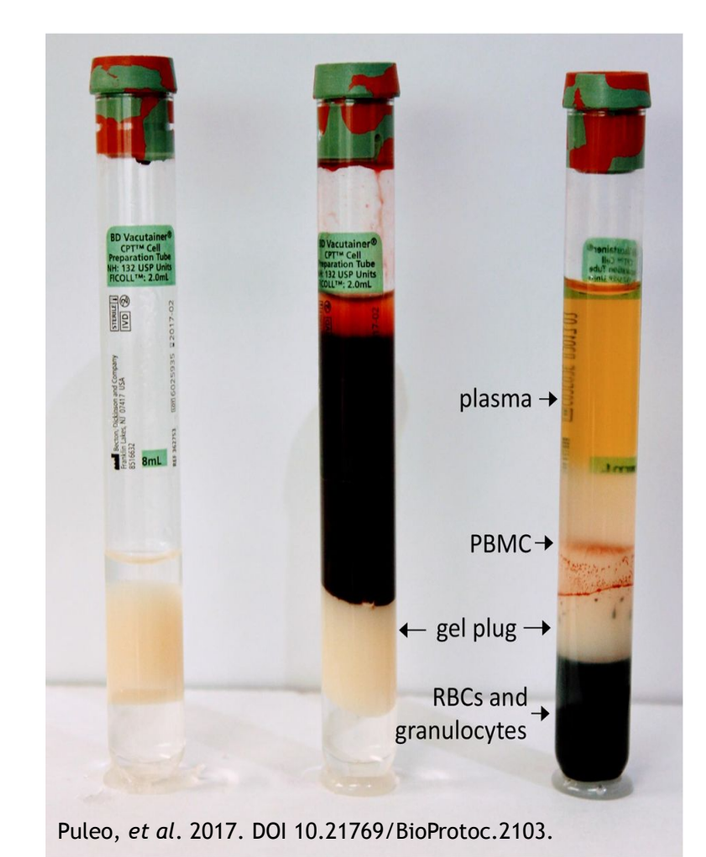
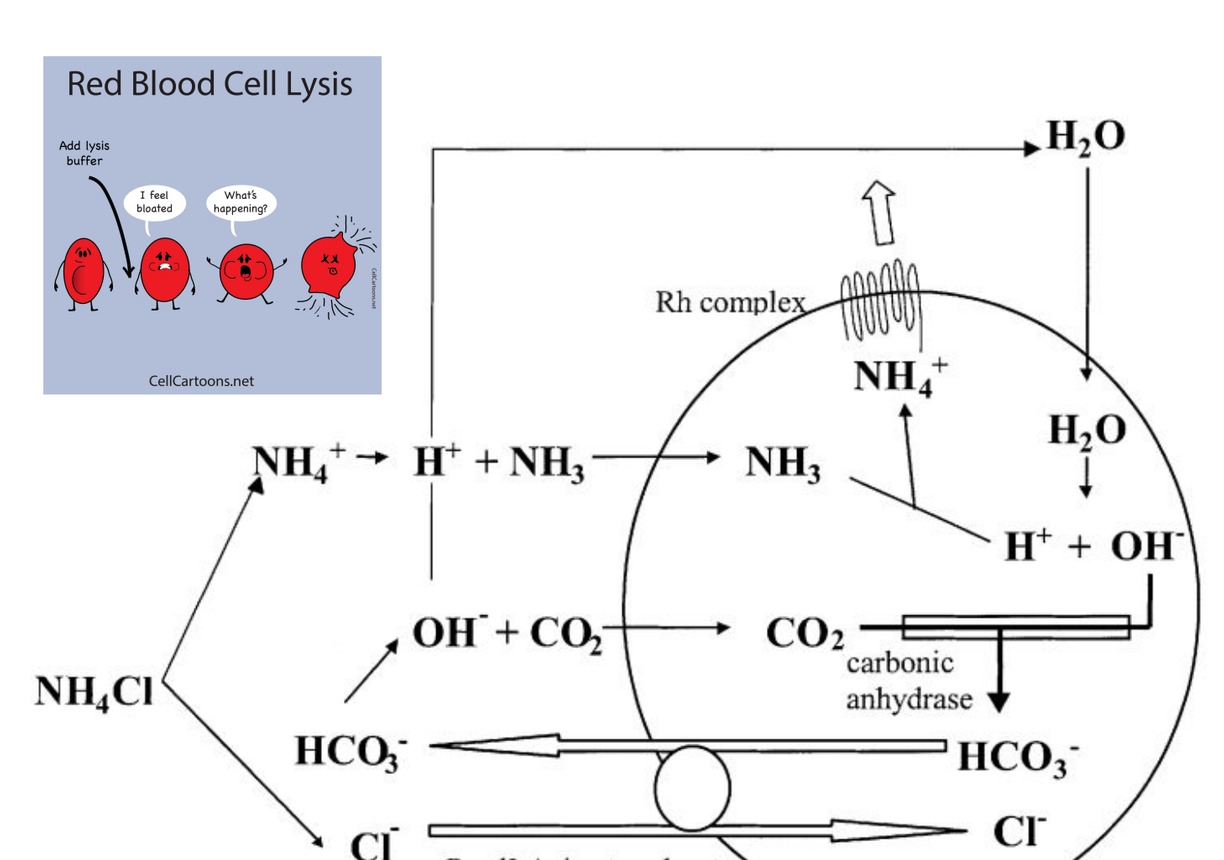


Figure 2: BD CPT Density gradient centrifuge tubes
BD CPT (cat no. 362753) come pre-filled with anticoagulant (sodium heparin or sodium citrate), a liquid density medium, and an inert gel barrier. Density Centrifugation of these tubes results in separation of RBCs and granulocytes from WBCs and plasma. This process isolates the WBC fraction known as peripheral blood mononuclear cells (PBMCs).

Ammonium-Chloride-Potassium Based RBC Lysis



Hemker, M.B., Cheroutre, G., Van Zwieten, R., Maaskant-van Wijk, P.A., Roos, D., Loos, J.A., Van Der Schoot, C.E. and Von Dem Borne, A.E.G.K. (2003), The Rh complex exports ammonium from human red blood cells. British Journal of Haematology, 122: 333-340. https://doi.org/10.1046/j.1365-2141.2003.04425.x

Figure 3: Mechanism of RBC Lysis
In vitro RBC lysis is mediated by Band 3, an anion transport protein in RBCs. Lysis occurs when RBCs are treated with an isotonic solution containing ammonium chloride (0.15 M), potassium bicarbonate(10 mM), and EDTA (0.1 mM). The buffer disrupts the osmotic equilibrium of the RBCs, causing an influx of chloride ions and efflux of bicarbonate ions, resulting in water influx and subsequent cell lysis. This method preserves the WBCs, yielding a purified suspension that includes granulocytes, which are typically 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.

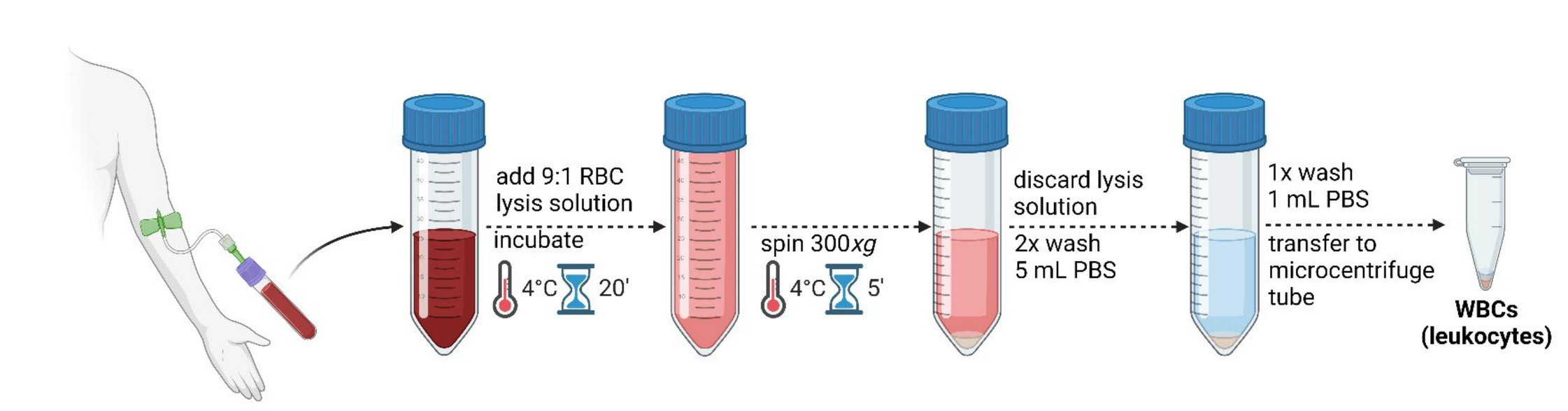


Figure 4: RBC lysis workflow. Lysis buffer (ammonium chloride potassium (ACK) solution, BioLegend catalog #420301) is added to the blood and incubated for 20 min at 4 °C. WBCs are isolated following two PBS wash steps and centrifugation to remove RBC debris.

Hypothesis & Methods

Visual Evidence of Incomplete Lysis During RBC Lysis Wash Steps

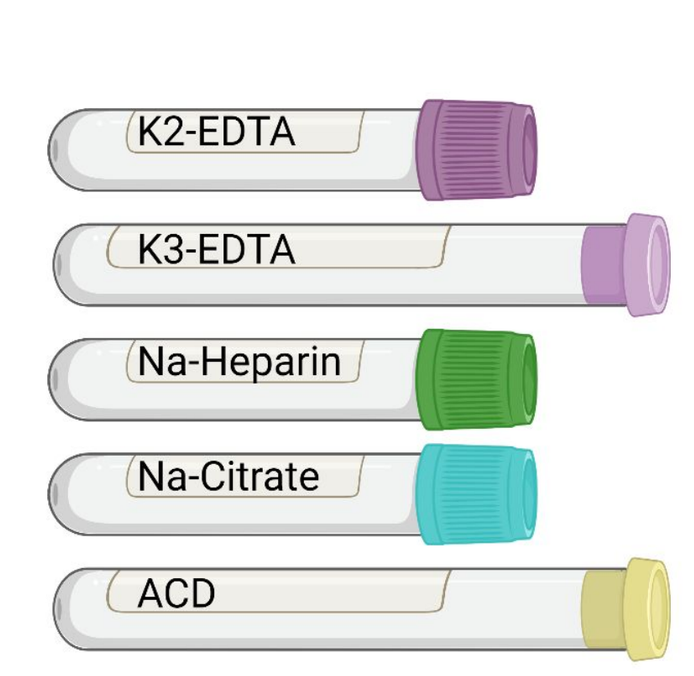


Figure 5: Common blood collection anticoagulants were selected to test the efficacy of the RBC lysis protocol.

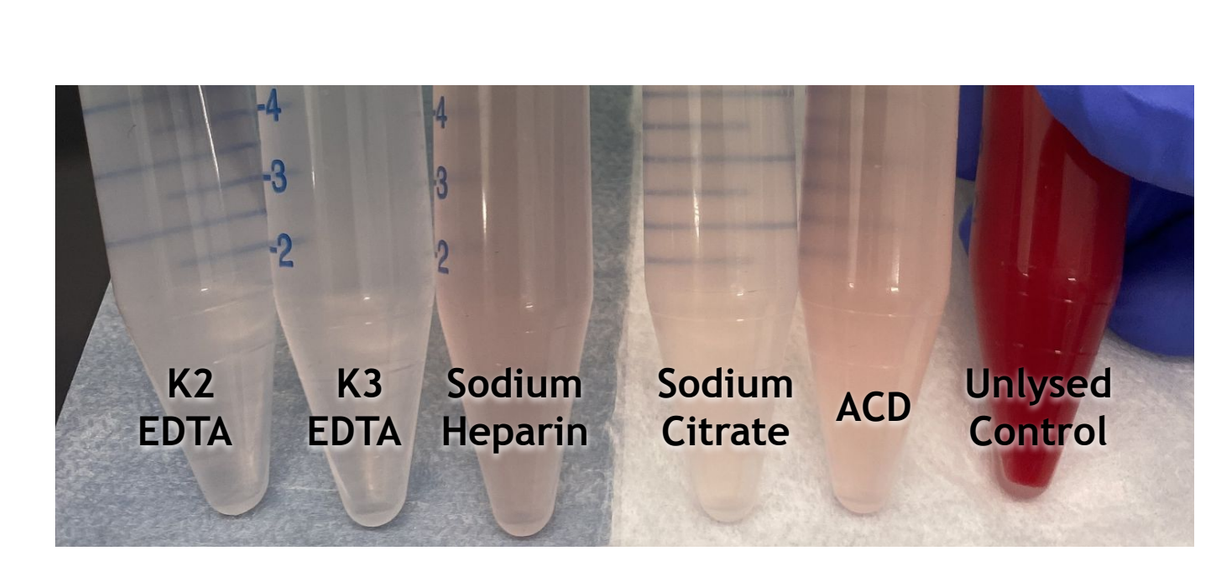


Figure 6: Incomplete lysis is visually apparent during the RBC lysis wash step. Sodium heparin, sodium citrate and ACD conditions displayed a noticeably pink coloration, in contrast to K2-EDTA and K3-EDTA.

We selected five common anticoagulants to compare the efficacy of the RBC lysis protocol (**Figure 5**). Incomplete lysis and subsequent RBC contamination is visually evident. **Figure 6** shows representative samples from each anticoagulant tube after lysis, during the wash step. In the unlysed control, lysis buffer is substituted with PBS. Blood samples collected in sodium heparin, sodium citrate and ACD exhibited more pronounced RBC contamination, indicated by a deeper pink hue, compared to samples from K2-EDTA and K3-EDTA tubes.

Liquid Chromatography Mass Spectrometry Workflow

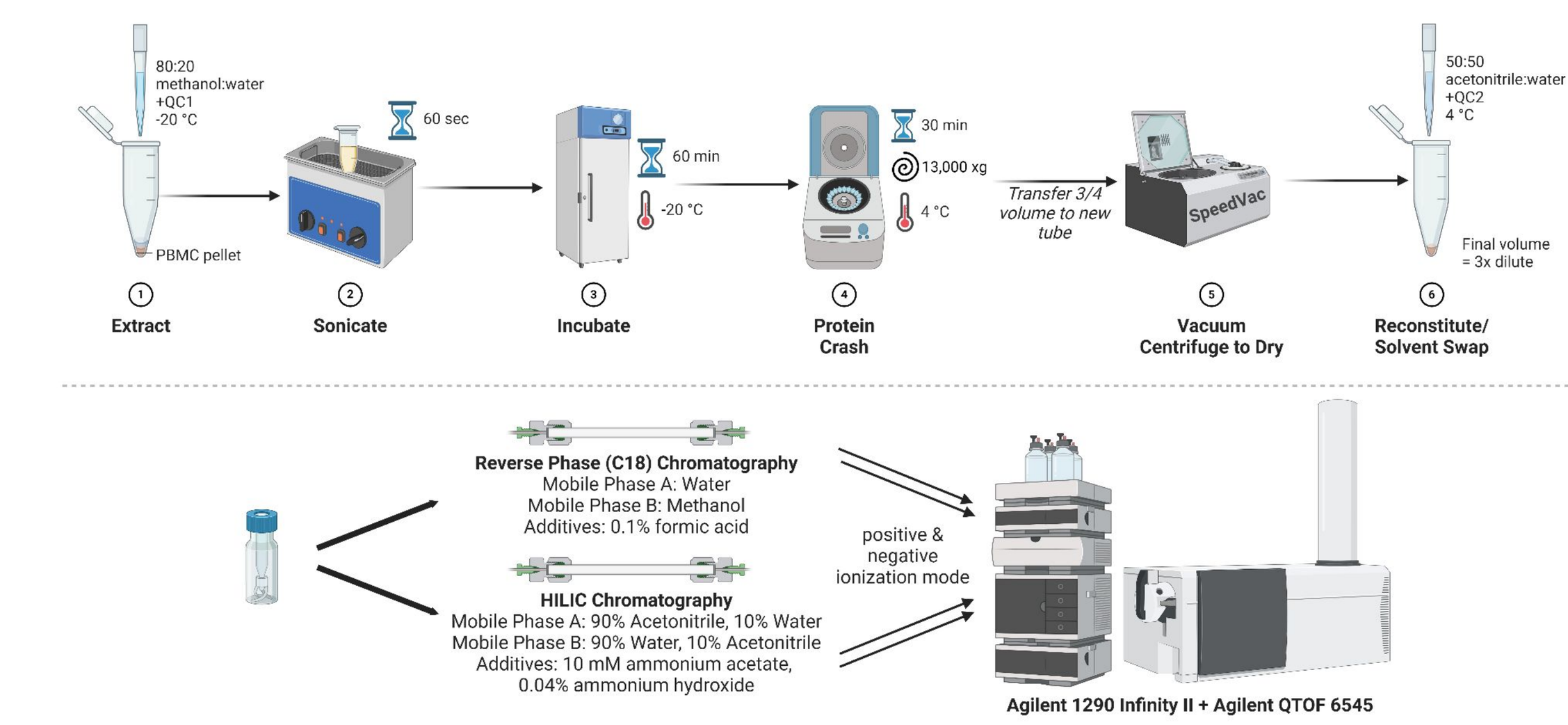


Figure 7: Metabolomics workflow for (top) metabolite extraction and (bottom) LC-MS analysis.

Untargeted Metabolomics (LC-MS)

Metabolic Markers for RBCs show Improved WBC Isolation via Lysis with EDTA Anticoagulant

Table 1: RBC markers from LC-MS positive ionization mode.

Compound	RBC peak area	RBC/CPT Intensity Ratio	RBC/Lysis Intensity Ratio	RBC/Plasma Intensity Ratio
Adenosine Triphosphate	1.07E+06	28	8.6	256
Aspartate	2.87E+04	2.1	2.5	1,283
Dimethyl Proline	2.91E+05	47	27	69
Ergothioneine	1.72E+05	34	5.8	49
Fructose 6-Phosphate	1.21E+04	89	5.9	7,633
GDP-Glucose	5.21E+03	1,432	2.7	4,021
Diphosphoglycerate	1.99E+05	25	25	61
Glutathione	1.29E+07	8.9	4.9	28,131
Glutathione Disulfide	8.97E+06	12	4.3	12,720
Glycerol 2-Phosphate	3.38E+03	169	4.0	74
Heme B	1.27E+05	9.6	3.7	4,327
Inosine Monophosphate	6.42E+05	6.4	1.2	22,962
NAD	5.85E+04	27	3.2	18,726
Nicotinamide	1.33E+06	6.2	4.6	43
Ophthalmic Acid	1.93E+05	5.2	5.1	877
Phosphoglycerate	1.38E+05	4.9	3.5	164
Protoporphyrin IX	2.75E+04	272	88	33
S-Adenosyl-Homocysteine	2.59E+04	6.6	1.3	22
S-Methyl-Ergothioneine	6.63E+04	104	15	91
Succinate	2.68E+04	26	5.9	178
Trimethyl-Lysine	3.01E+05	70	34	35
Trimethyl-Tyrosine	8.62E+04	29	11	39
Uridine Diphosphate	2.61E+04	13	2.2	9,379

Whole blood collected into vacutainers was fractionated into plasma and RBC portions via centrifugation. PBMCs were isolated using either density gradient centrifugation or ACK lysis. All samples were extracted and analyzed using the protocol described in **Figure 7**. Statistical analysis was used to identify features that are upregulated in the RBC fraction, and matching against our internal LC-MS libraries provided compound identifications. **Table 1** describes select RBC markers from the positive ionization channels, including the peak areas for the RBC fraction and peak intensity ratios for RBCs against CPT-PBMCs, Lysis-PBMCs, and plasma.

Many metabolites that we identified as RBC markers have been previously reported in literature as enriched in RBCs, and we additionally see excellent agreement when using our methods with previously reported RBC/plasma ratios for specific compounds (e.g. ophthalmic acid, trimethyl-tyrosine).[5]

[5] Chaleckis, Romanas et al. "Individual variability in human blood metabolites identifies age-related differences." *Proceedings of the National Academy of Sciences of the United States of America* vol. 113,16 (2016): 4252-9. doi:10.1073/pnas.1603023113

The metabolites identified in Table 1 act as potential markers for RBC contamination in processed blood samples. One of these markers is **heme B** ($C_{34}H_{32}O_4N_4Fe$, m/z 616.177 (fixed positive charge)), a compound found in hemoglobin. **Figure 8** exemplifies how heme B identification via LC-MS may be used as a quantitative marker for RBC contamination in PBMC preparations. The % RBC contamination was calculated in a linear association by dividing the heme B signal of lysis samples by the average intensity from RBC samples, multiplied by a slope correction factor. While each laboratory must determine this slope correction factor individually, it is readily determined from a LC-MS calibration curve of heme B, or as shown in this case, a linear regression with a technology such as FACS. It is important to recognize the sigmoidal nature of LC-MS concentration-intensity curves; *we caution against assuming linear relationships for LC-MS fold changes and analyte concentrations without prior quantitative vetting.*

The approach described above can be applied not only to RBCs, but to other components of blood such as plasma and granulocytes. **Figure 9** shows a Venn diagram detailing the number of unique LC-MS features found in various blood components for our HILIC/positive mode channel. The 290 metabolites in the RBC portion of the diagram include many of the markers from Table 1, such as nicotinamide and phosphoglycerate. Furthermore, because PBMC isolation using CPT density centrifugation tubes removed granulocytes along with RBCs, the list of 156 features unique to Lysis-prepared WBCs offers a list of potential granulocyte markers.

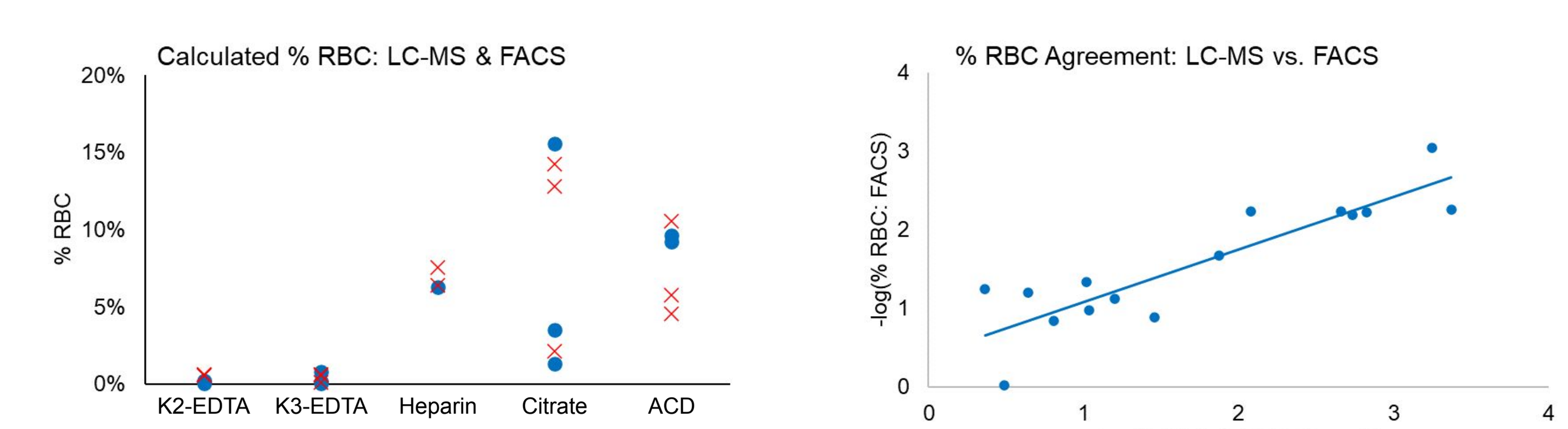


Figure 8: Quantitative analysis of RBC contamination using the LC-MS results for Heme B. Calculated percent RBC contamination from Heme B LC-MS intensity is plotted against % RBC as determined by FACS analysis (left). The log-log plot of % RBC as determined by FACS against % RBC as determined by Heme B LC-MS profile illustrates the high correlation between the two methods (right).

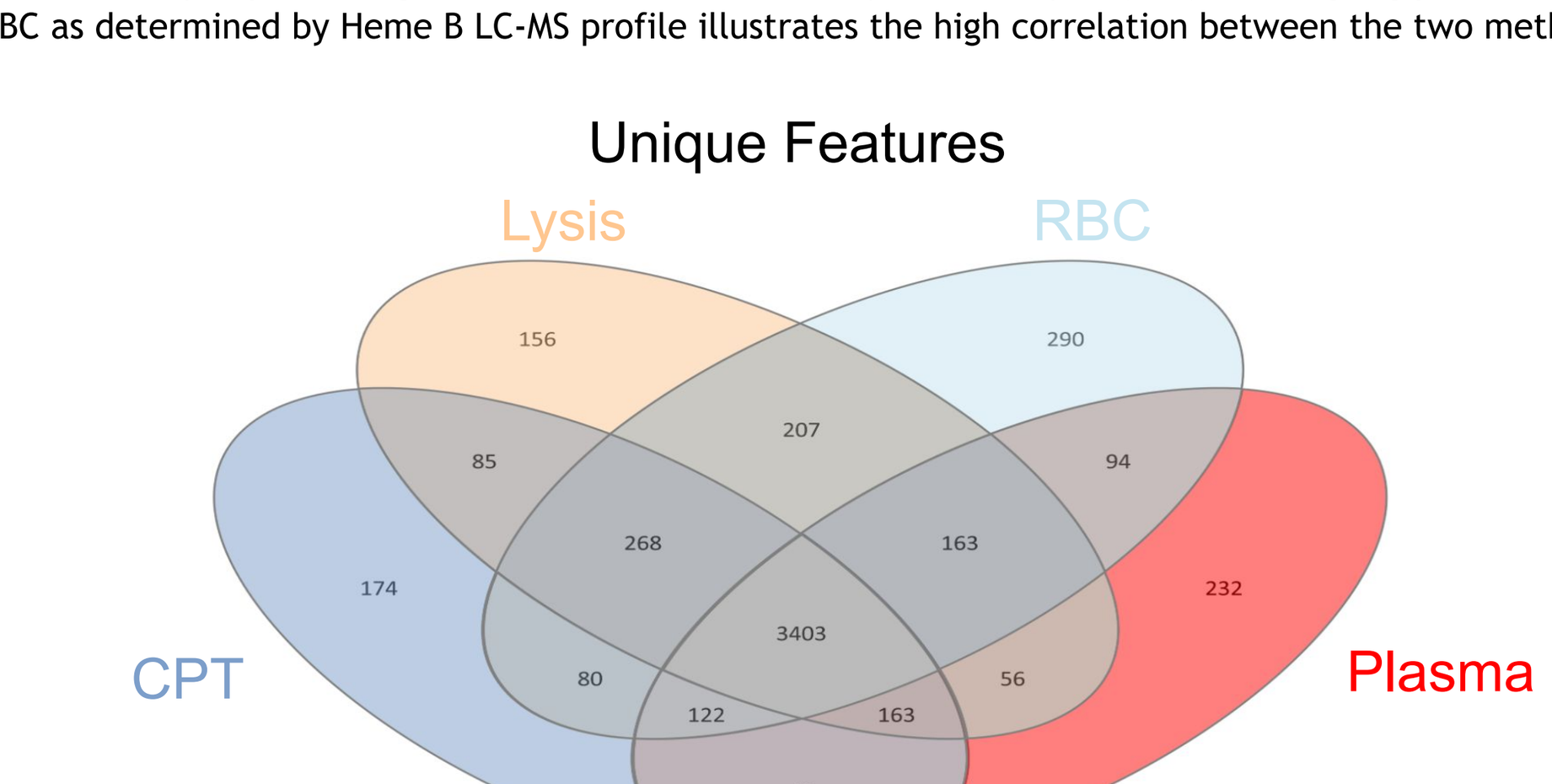


Figure 9: Venn diagram of unique features as identified by untarged LC-MS metabolomics on our HILIC/positive ionization mode channel. The four blood fraction groupings include RBCs, plasma, PBMCs prepared by RBC lysis, and PBMCs prepared by CPT density centrifugation.

Surface Marker Staining Workflow for Imaging and FACS Analysis

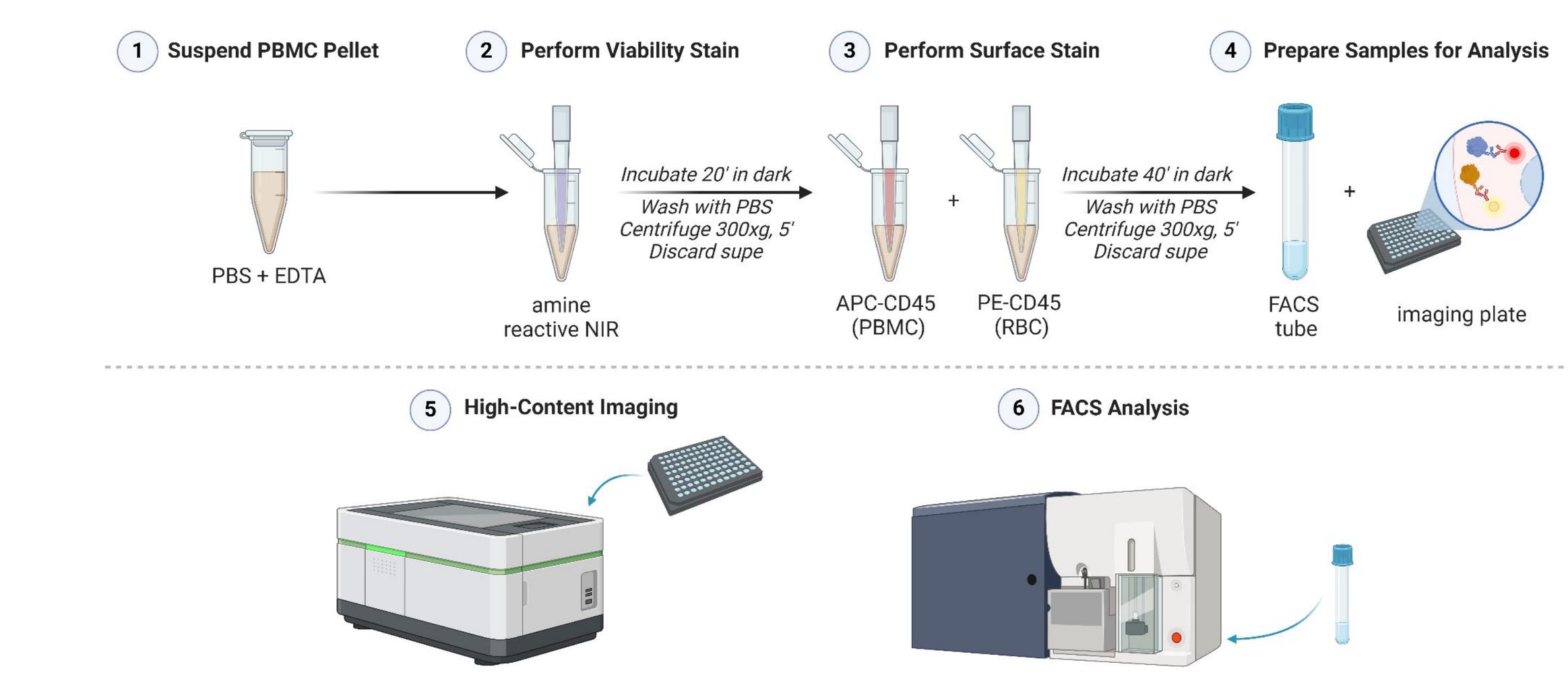


Figure 10: Surface marker staining protocol for imaging and/or flow cytometry analysis. Imaging was performed using a Revvity Operetta CLS High-Content Analysis system and FACS analysis used a BD FACS Melody Cell Sorter.

Microscopy Using High-Content Imager

Imaging Analysis Development with RBCs Spiked into CD45+ Cell Culture (Jurkat cells)

In the interest of further characterization and interpretation of FACS results, we aimed to develop a microscopy workflow. Fluorescent imaging provided an opportunity to gather additional information, while utilizing the same surface staining protocol used for FACS analysis. Furthermore, this approach provides the benefit of being non-destructive and can be employed to analyze samples prior to FACS analysis. To trial this protocol, we spiked RBCs collected from fingerstick blood into cultured Jurkat cells, which stain positive for CD45 and therefore function as WBC representatives. The mixed samples were stained with APC-CD45 and PE-CD235a concurrently as described in **Figure 10** and then analyzed via imaging and FACS.

The presence of RBCs was determined as percent of CD235+ events in both imaging and FACS analysis. **Figure 11** shows a calibration curve style plot of Jurkat cells spiked with RBCs. **Figure 12** displays the collection of images from which the RBC percentage was determined.

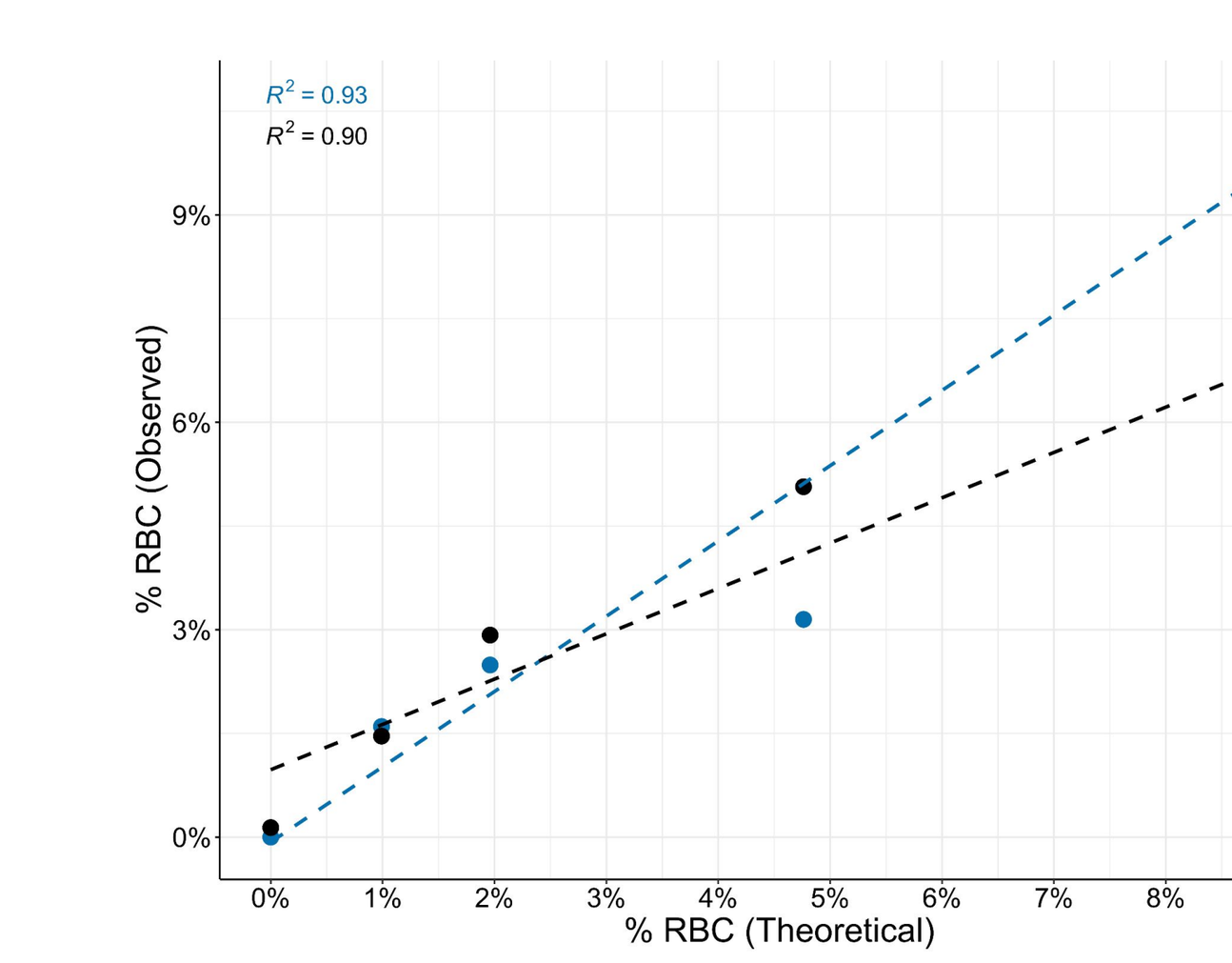


Figure 11: Calibration curve for quantification of RBC contamination using CD235 and CD45 staining, as determined by imaging and FACS.

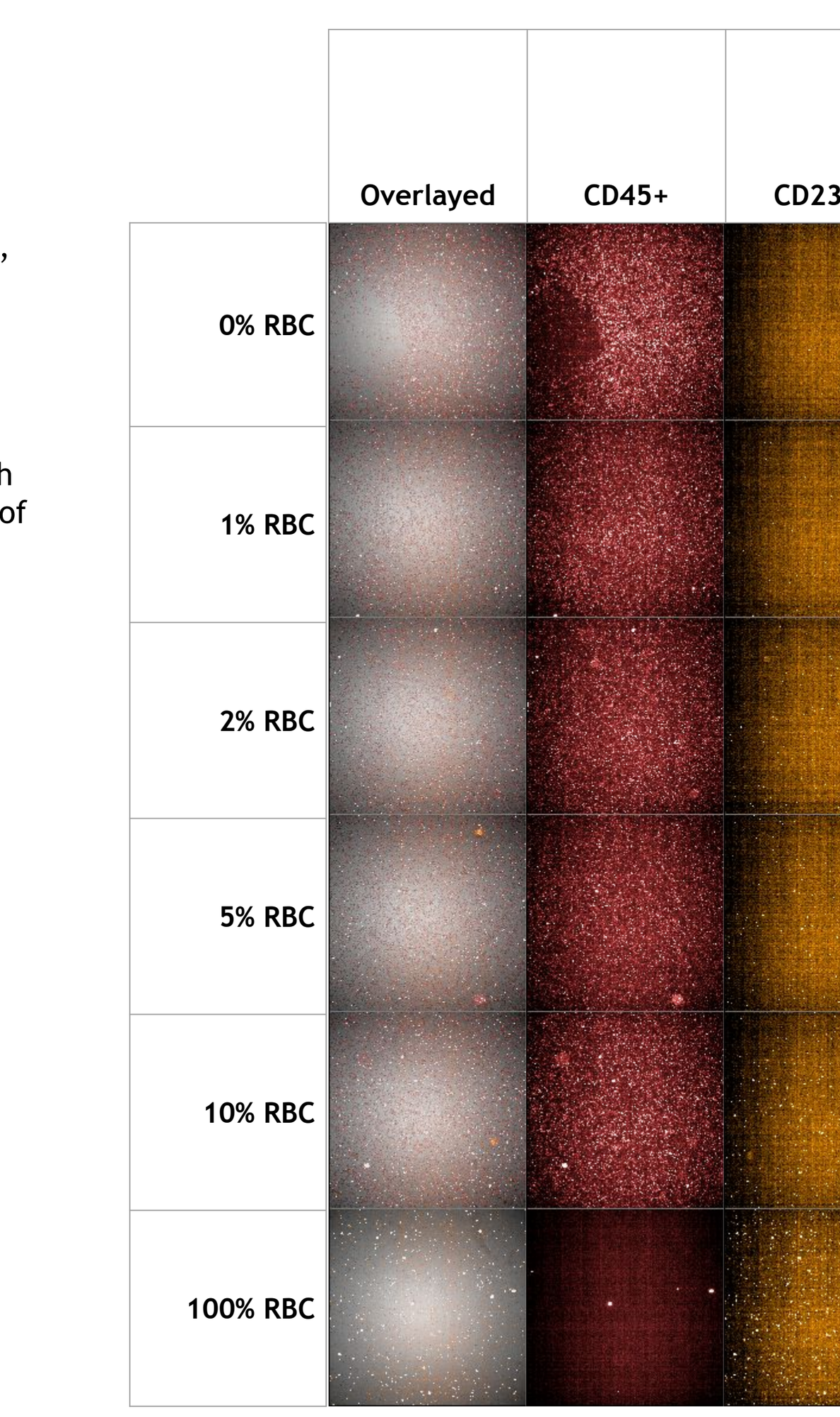


Figure 12: Cultured Jurkat cells (CD45+) spiked with varying concentrations of RBCs (CD235+).

Flow Cytometry Analysis (FACS)

Quantification of Cellular Populations Using FACS Analysis Affirm LC-MS Metabolomics

We chose to use FACS to quantify the populations of RBCs and WBCs to complement metabolomics results and further investigate the impact of blood collection tube on the efficacy of RBC lysis. WBC suspensions isolated from whole blood collected into the five anticoagulants were characterized after depletion of RBCs using ACK lysis. CD235a+ labeling indicates the presence of contaminating RBCs that were not lysed and removed during preparation, whereas CD45+ labeling verifies the WBC population.

FACS results confirm the visual observations that using sodium heparin, sodium citrate, or ACD anticoagulant tubes for initial blood collection results in reduced efficacy of the RBC lysis step. Non-EDTA anticoagulants tested produce a WBC suspension that is contaminated with higher amounts of RBCs (**Figure 13**).

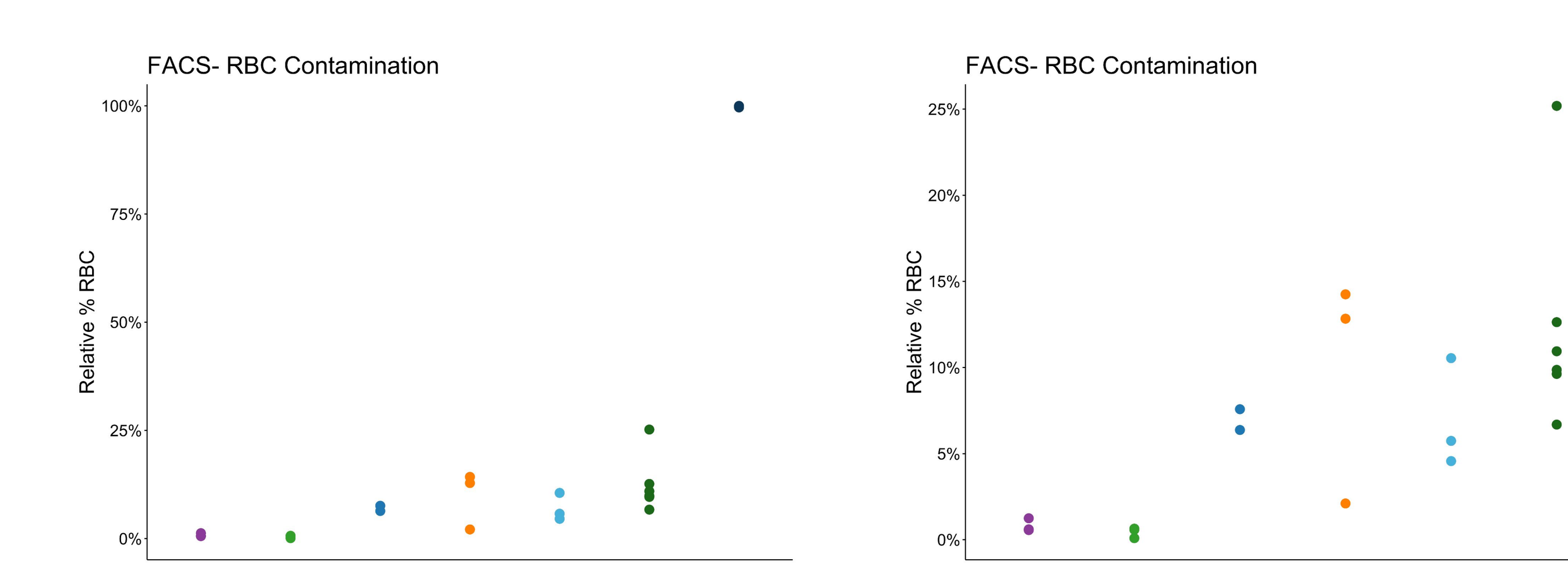


Figure 13: K2-EDTA and K3-EDTA anticoagulant produce the lowest levels of RBC contamination.
Residual red blood cell presence indicated by CD235+ labeling using FACS analysis on all tested anticoagulants, CPT tubes, and unlysed controls (left) and scaled y-axis showing lysis and CPT conditions (right).

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