RBC Lab Newsletter May 2023

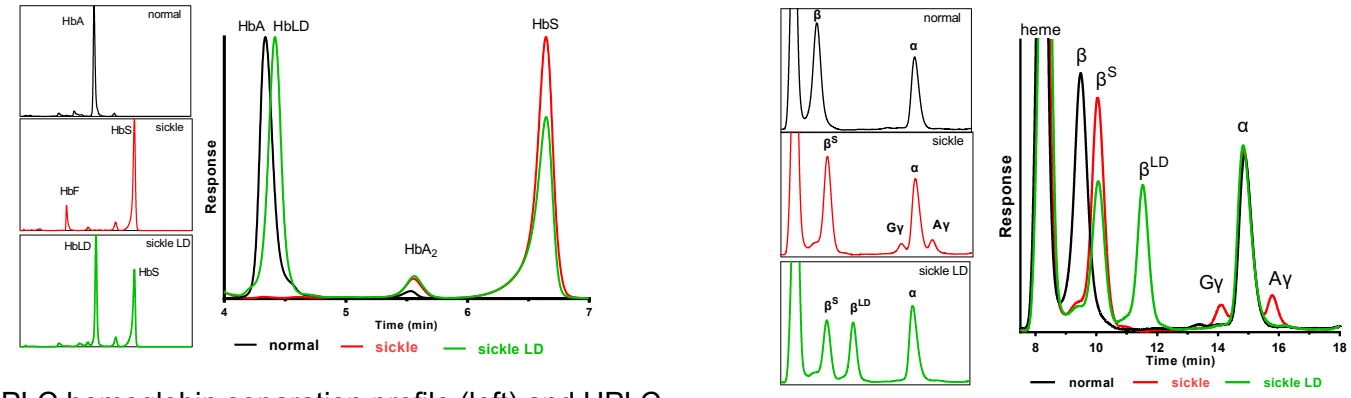
The Red Blood Cell (RBC) lab continues to develop new assays or improve existing technologies to better support studies in hemoglobinopathy research. In this newsletter we present an improved **hemoglobin and globin assessment** by HPLC, a **rapid screen for oxygen affinity** shifting compounds, and a quantitative approach to assess **hemoglobin F and sickling**. Please contact us for additional information.

Hemoglobin and globin assessment.

The HPLC assessment of hemoglobin and globin in the RBC Lab is superior, as shown by two examples below: hemoglobin variants and hemoglobin modification.

Hemoglobin variants:

Hemoglobin La Desirade (HbLD) is a hemoglobin variant characterized by amino acid Alanine (Ala) replacing Valine (Val) at position 129 in the beta globin chain. In both standard Electrophoresis (CE) and HPLC, HbLD runs like normal hemoglobin A (HbA). This poses a diagnostic problem. When combined with sickle hemoglobin (HbS), patients with HbLD/S could be misdiagnosed as sickle cell trait (HbA/S or SCT). SCT cells will sickle under low oxygen and sickling is also reported for HbLD/S. The rate of sickling may differ as the levels of HbS in these HbLD/S heterozygotes is significantly increased as compared to SCT. This suggests that HbLD/S poses a higher risk for sickling which may become apparent at high altitude (lower partial pressure of oxyegen, PO_2).
In contrast to published data on HPLC and clinical lab standard CE analysis, the RBC Lab HPLC hemoglobin and globin assessment allows separation of HbLD from HbA, which is further confirmed by the detection of an altered beta globin species. Only 10 µl of fresh or frozen blood is needed for these assays.

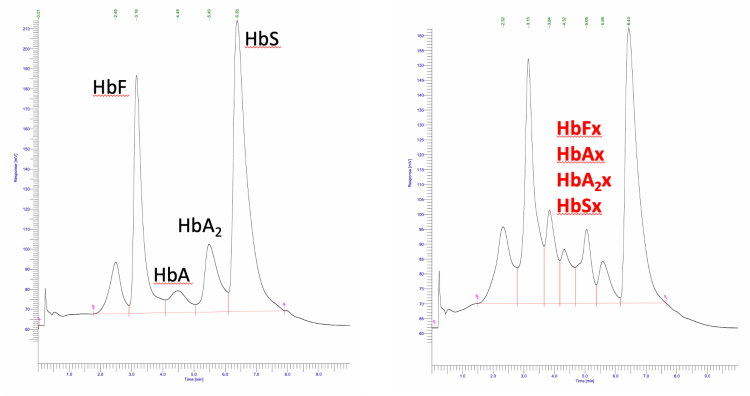


HPLC hemoglobin separation profile (left) and HPLC globin separation profile (right) of blood from a sickle cell disease (HbSS) patient in red, normal (HbA) in black, and HbLD in green.

Hemoglobin modification

Drugs designed to modify globin chains of Hb can be detected and quantified. The example shows data from patients on treatment with Voxelotor.

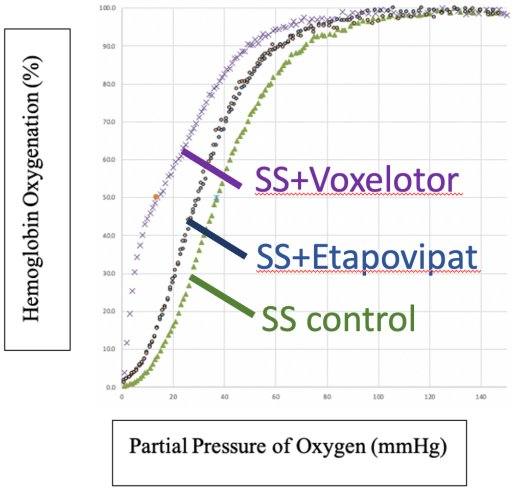
Modification of their Hb species, can be assessed by HPLC on small amounts of blood equivalent to a finger prick.

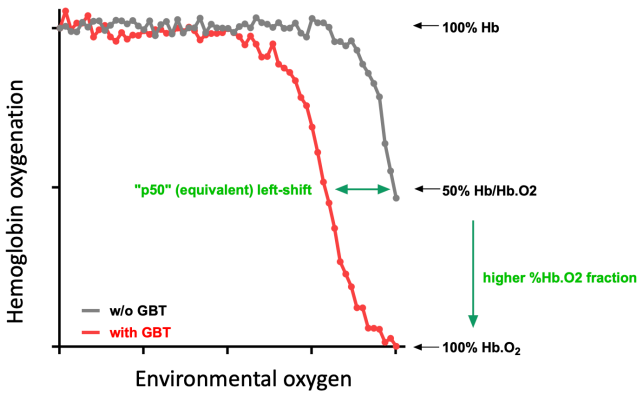


Before voxelotor On voxelotor

Rapid Screen for Oxygen Affinity

The RBC lab uses the Hemox analyzer to define oxygen affinity. While this technology provides an important assessment, the Hemox analyzer is not a practical way to evaluate/screen large sets of compounds (libraries) with the potential to shift RBC oxygen affinity. The RBC lab has developed a rapid high throughput screening test to define the oxygen affinity shifting of large numbers of potential compounds. The figure shows the typical Hemox curves on the left and the status of RBC hemoglobin oxygenation in two wells of a 96 well plate on the right. The spectroscopic characteristics of hemoglobin are followed in time while the environmental availability of oxygen is changed. As expected, treatment with Voxelotor shows a clear shift to an increased oxygen affinity as compared to untreated RBC. Using this microplate assay allows the evaluation of large numbers of compounds in their ability to shift RBC oxygen affinity in a single experiment. The Hemox analyzer can be used to confirm the change in the oxygen affinity curve of potential compounds identified by the high throughput screening

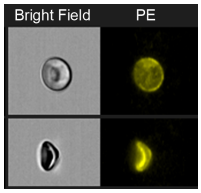


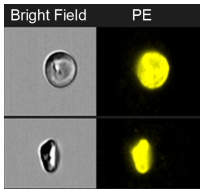


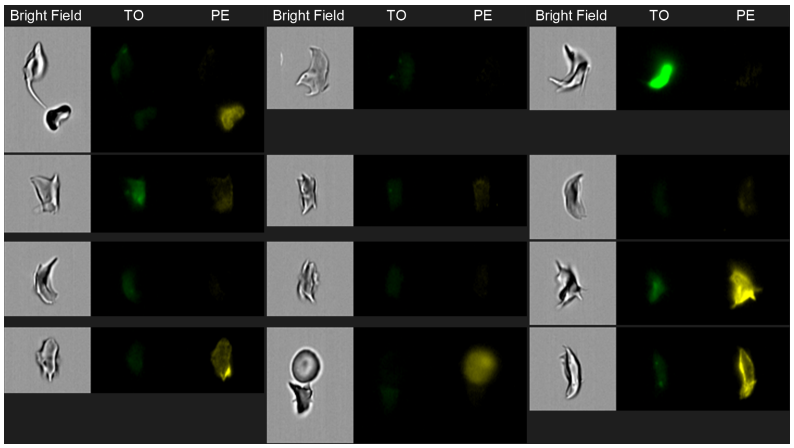
LEFT:HEMOX analyzer assessment of RBC incubated with voxelotor or etapovipat, a pyruvate kinase stimulator.
RIGTH: HTS Comparison of small amounts of RBC, either voxelotor treated (red) or untreated (grey) in two wells of a 96 well plate.

Hemoglobin F cells and sickling
Hemoglobin F (HbF) reduces the sickling activity of the sickle RBC and improves the quality of life of sickle patients. Assessment of HbF cells is an important parameter in evaluating the effect of HbF stimulators.

The RBC lab uses image flowcytometry to detect and quantify the population of reticulocytes and adult RBC that contain HbF. The presence of HbF (yellow) in adult RBC and Reticulocytes (Green) can be shown in thousands of cells starting with 5 µl of blood.







The RBC Lab at UCSF builds on decades of experience in DNA, RNA, proteins, and metabolites analysis of all blood cells and plasma in academic and clinical studies. Our experienced staff partners with the clinic, academia and industry to evaluate and implement projects aimed to improve the quality of life of patients with abnormalities in blood and blood cell physiology.

What defines the RBC lab:

- We partner with academia and industry in blood related research studies.
- We take on projects that cannot be accomplished in the clinical lab.
- We assist in the design of clinical trials, sample collection, shipment, data collection, and analysis.
- We use a vast panel of assays and unique instrumentation to define the levels of components in the complex pathways of blood physiology.
- We develop new technologies to measure and validate assessment of blood components.
- We support the definition of newly discovered abnormalities such as novel hemoglobin mutations.
- We provide the use of murine models of human disease in the development of human clinical trials.
- We provide mentorship and training of fellows
- We provide mentorship and lab experience for high school and college students in the UCSF summer student program.

Contact Us

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Technology overview
Assays and technologies indicated below are used by the RBC lab to define pathology and is used to set biomarkers to assess the success in clinical trials. In a general sense a shift towards normal is the goal of treatment, either by drugs or gene therapy, and monitoring these biomarkers in time by the RBC lab have contributed to the currently available treatment of hemoglobinopathy patients.

Advia 2120
The advia 2120 hematology analyzer provides validated data on **human** an **murine** blood including Blood hemoglobin, RBC count, RBC volume, RBC hemoglobin, RBC hemoglobin concentration, Reticulocytes, Platelets, and WBC differential. We use a custom developed assessment of the density distribution in the RBC population, important to define the risk of sickling. The use of murine models of diseases such as sickle cell and thalassemia have been pivotal in preclinical trials for the development of drugs and gene therapy. In contrast to clinical lab equipment we have validated assays to assess murine blood. In all cases a shift towards normal is aimed and the Advia 2120 provides this assessment.

Hemoglobin assessment
The RBC lab uses HPLC technology to determine:

- Hemoglobin species (HbA, HbF, HbS,HbE,HbA2, HbH)
- Hemoglobin modification
- Globin species (Alfa,Beta,Gamma globin)
- Globin modification
- Heme released from hemoglobin

Oxygen affinity
The ability to bind oxygen in the lungs and offload it to the tissues is the major role of red blood cells. The hemox analyzer provides data on the binding and release of oxygen at different oxygen tensions. Hemoglobin types as well as red cell metabolism (glycolysis) define oxygen transport. Modification of oxygen affinity is used to reduce sickling and forms the basis for several currently ongoing clinical trials.

Flowcytometry and Image flowcytometry
Flowcytometry is used to define cell surface markers as well as specific markers inside individual cells. Image flowcytometry combines flowcytometry with microscopy providing image analysis of many thousands of cells in a short time. As an example we use this technology to measure HbF in RBC and reticulocytes, show the presence of phosphatidyl serine (PS) on the surface of the cell and the binding of DNA present plasma.

Increasing HbF cells are an important strategy improving the quality of life of hemoglobinopathy patients. PS normally on the inside of the membrane is exposed in apoptosis or programmed cell death and provides an early signal for cell removal. PS exposure of platelets is essential hemostasis but will contribute to thrombosis when PS exposing cells are not removed. Cell free DNA needs to be removed by binding to RBC to lower an upregulation of the inflammasome.

Sickling assay
The RBC lab has developed a unique way to measure the morphology changes of sickle RBC exposed to low oxygen tension in time. The formation of individual “sickled” cells in time provides insight in the kinetics of sickling. Treatments that lower the rate of sickling are used to improve the quality of life of sickle cell patients. These treatments include hydroxy urea, compounds that increase oxygen affinity and gene therapy.

Enzymes and intermediates
Factors involved in blood chemistry as well as RBC glycolysis are measured using commercially available kits as well as custom developed assays. Manipulating RBC glycolysis is the approach used by Forma and Agios to increase oxygen affinity by decreasing 2,3 DPG levels by increasing pyruvate kinase activity. Several

pathways are affected in the RBC and the RBC lab provides assays to measure both activity and specific activity of key components. Plasma factors such as cell free hemoglobin, heme, cytokines and secretory phospholipase A2 play key roles in blood pathology and normalization is the key to determine effectiveness of treatment.

RBC deformability

Ektacytometry exposes RBC to shear stress in a Couette viscometer. This allows assessment of RBC deformation at different osmolarities, oxygen tensions and exposure to oxygen free radicals under shear stress, an environment that mimics the conditions experience by RBC in the circulation.