

Hemoglobinopathy and thalassemia detection

Traditional methods and a novel method — capillary electrophoresis technology

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In humans, two pairs of unlike globin chains combine with four heme groups to form hemoglobin (Hb), a protein that is carried by red cells and picks up oxygen in the lungs and delivers it to the peripheral tissues. One of the globin chain pairs in hemoglobin is always alpha (with the exception of the very first weeks of embryogenesis), while the second pair is “non-alpha.” That is, it can be made of beta- (β), delta- (δ), or gamma (γ) chains.

In the healthy newborn, Hb F ($\alpha_2\gamma_2$) is the major hemoglobin (~75%). Fetal hemoglobin (Hb F) is replaced by Hb A ($\alpha_2\beta_2$) and Hb A2 ($\alpha_2\delta_2$) during the first six to 12 months of life. In healthy adults, hemoglobin is comprised of Hb A (~97%) and Hb A2 (~2.7%), with only trace amounts of Hb F, if any.

Two types of disorders may affect globin chains—qualitative and quantitative. Qualitative disorders, i.e., hemoglobinopathies, result from any of the following: i) substitution of one amino acid for another (as in Hb S and Hb C); ii) deletion of a portion of the amino acid sequence (as in Hb Gun Hill); iii) abnormal hybridization between two chains during meiosis (as in Hb Lepore); and iv) abnormal elongation of the globin chain (as in Hb Constant Spring). Obviously, any alterations listed above lead to changes in molecule structure or charge and they can be detected with the appropriate methodology.

Currently, over 1,400 Hb variants are listed in the globin chain database, with the majority of them being beta chain variants.

Thalassemias are quantitative disorders affecting the rate of otherwise normal hemoglobin synthesis. The β -thalassemia carrier state is a benign condition with mild anemia, red blood cell hypochromia and microcytosis, and an elevated Hb A2 level. In comparison, severe disease (β -thalassemia major) requires lifelong blood transfusions and chelation therapy.

Since increase in Hb A2 concentration is indicative of beta-thalassemia, it is useful to obtain an accurate relative Hb A2 value. With many testing methods, common Hb variants can interfere with an accurate Hb A2 quantitation. It is also important to account for delta chain Hb variants in the sample; if any are present, the delta chain variant concentration (percentage) must be added to the Hb A2 value to obtain an accurate total Hb A2 concentration.

Alpha-thalassemia affects the synthesis of alpha globin chains, and the severity of disease is dependent on the extent of gene deletion. Loss of two out of four alpha-chains encoding genes results in an α -thalassemia trait, characterized by microcytosis with little or no anemia. Loss of

three genes results in Hb H (4 β chains) disease, a moderate hemolytic anemia, while loss of all four genes is incompatible with independent life.

Electrophoretic hemoglobin separation methods

Electrophoresis has long been the method of choice in hematological laboratories for qualitative and quantitative hemoglobin analyses. Currently, four different techniques are routinely used in the lab setting: 1) alkaline and acid gel electrophoresis; 2) isoelectric focusing (IEF); 3) high-pressure liquid chromatography (HPLC); and 4) capillary electrophoresis (CE).

Sebia Electrophoresis provides multiple platforms for the detection of hemoglobinopathies and thalassemias—fully automated capillary electrophoresis systems and a semi-automated agarose gel system to accommodate both alkaline and acid agarose gel electrophoresis.

Alkaline and acid agarose electrophoresis

Because of its simplicity, alkaline gel electrophoresis is one of the most popular methods for Hb screening. Semi-automated agarose gel electrophoresis is also cost-effective for low- to medium-volume laboratories. However, the technique is relatively laborious, requiring manual sample preparation. Red blood cells must be washed in saline to remove plasma proteins and to eliminate non-hemoglobin bands on the gel. Electrophoresis at alkaline pH (8.5) allows for the separation of the major hemoglobins and a number of less common Hb variants. Visualization of the Hb bands is done by automated staining of the gel with amido black. The clear background of the gels enables measuring the concentration of individual fractions by densitometric scanning. However, due to the precision and accuracy of Hb in low concentrations (Hb A₂ for example), the College of American Pathologists (CAP) no longer recommends the use of densitometric scanning for quantification of Hb A₂. With alkaline agarose gel testing, some common Hb variants comigrate, such as Hb C, Hb E, Hb A₂ & Hb O-Arab and Hb S, Hb D and Hb G.

In order to separate some Hb variants that commonly comigrate at alkaline conditions, the sample may also be analyzed on gel at an acidic pH (6.0). In these conditions, molecular charge will differ and migration patterns will change. As a result, Hb S can be differentiated from Hb D, and Hb C can be differentiated from Hb E.

Isoelectric focusing

IEF provides excellent separation of many hemoglobin variants and detects fast-migrating or low concentration hemoglobin variants such as Hb H, Hb Bart's, and delta chain variants.

IEF gels contain special molecules—ampholytes—that create a pH gradient in an electrical field. When a pH gradient is present, hemoglobin molecules migrate to a position on the gel where the net charge equals zero (0), resulting in very narrow and focalized bands. On IEF gels, Hb C separates from Hb E and Hb O-Arab, and Hb S from Hb D and Hb G. IEF gels, however, are processed manually and require a significant amount of technical time. Additionally, IEF results are qualitative, and interpreting results requires significant experience.

High pressure liquid chromatography

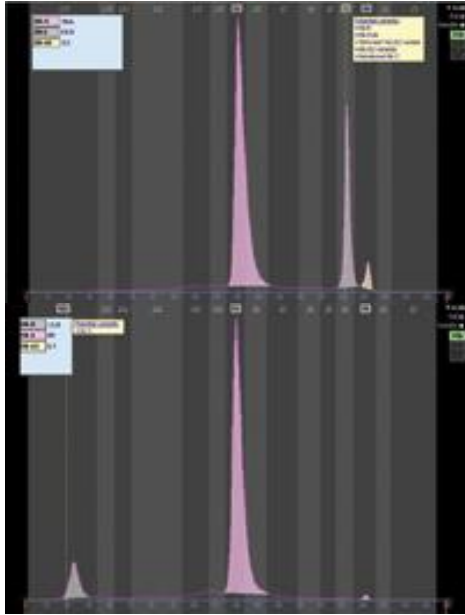
HPLC is a pressure-driven technique. Hemoglobin samples are injected into a resin column and retained based on the charge. The eluting solution that competes for the negatively charged resin is added in increasing concentration. Hemoglobin variants elute from the column and are detected at 415 nm, then at 690 nm to correct the baseline of the result. The hemoglobin retention time (from injection until the maximum point of each peak) is calculated and plotted on a chromatogram.

HPLC instruments are primarily indicated for the measurement of Hb A2 and F, but also provide data (retention times) on many Hb variants. However, HPLC should not be used as the sole method for identification of hemoglobin variants.¹ HPLC is very complementary to CE technology; together these two automated methodologies provide valuable data for result interpretation.^{2,3} HPLC techniques result in patterns that are relatively complex and require training and experience for interpretation of results.⁴

Capillary electrophoresis

CE technology utilizes liquid flow electrophoresis—buffer replaces agarose gel as the medium. Hemoglobin variants are separated by electroosmotic flow at an alkaline pH (9.4) using negatively charged silica capillaries and high voltage. Multiple samples undergo an eight-minute high-resolution separation, concurrently. A high-resolution hemoglobin separation is obtained, similar to IEF separation. The ideal wavelength of 415 nm is utilized for hemoglobin detection with CE. The result, or electropherogram, is made up of 300 consecutive readings (dots) and is divided into 15 zones. To facilitate interpretation, results are automatically positioned with regard to the Hb A and Hb A2 fraction in the sample. Hemoglobins (normal and variant) are displayed as peaks, and the zone to which a variant belongs is identified automatically by the system. An on-board hemoglobin library is present in the form of a drop-down list and lists all of the normal and variant hemoglobins that may be present within a particular zone.

With Sebia's CE systems, packed red blood cell samples are utilized for analysis. Plasma is removed from samples, and the bar-coded primary sample tube is loaded onto the instrument; all other steps in sample processing and separation are performed automatically by the system.



More features of CE technology

- In one analysis, separation of Hb S from Hb D, and Hb C from Hb E (and from Hb A2).
- Precise, quick quantification of Hb F and Hb A2, even in the presence of Hb S.⁵
- Posttranslational Hb variants (such as glycated HbS1c) do not separate from the main fractions.⁶
- Delta chain variants, alpha chain variants, and other minor Hb fractions are readily visualized.⁸
- Hb H and Hb Bart's are more readily detected and measured by CE than by the HPLC method.⁴

Multiuse instrument

Sebia systems may be used for other types of analysis, including serum/urine protein electrophoresis, immunotyping (automated immunofixation alternative), and CDT (a marker for chronic alcohol abuse)⁸

CAP requires the use of a second, complementary technique for abnormal hemoglobin results. CE is most complementary with acid gel electrophoresis and HPLC. By combining CE and HPLC methodologies, one can significantly reduce the number of unusual hemoglobin variants that can be confused with normal hemoglobins or common Hb variants (2).

Newborn screening with dried blood spot samples

Sebia's newest FDA-cleared CE assay for hemoglobinopathy testing is Capillarys Neonat Hb Fast. It is used for the screening of newborn blood samples collected on Guthrie Cards. Newborn dried blood spot samples are screened for the presence of normal hemoglobins (F and A) and common hemoglobin variants to include S, C, D, E, and Bart's. The system is fully automated and fast, with an instrument throughput of 96 results in two hours. The fast throughput is

accomplished due to eight simultaneous analyses taking place; a high-resolution seven-minute migration occurs for each newborn sample with results similar to IEF separation. Result interpretation is aided by automatically color-coded curves (normal or abnormal results) and on-board hemoglobin library by zone. All normal hemoglobins and common variants migrate in different zones—Bart’s, A, F, D, S, E, A2, and C.

The following table lists HPLC migration characteristics in the presence of common variants.

Hb present	Hb A2 result	Hb F result	Comments
S	falsely elevated		coelution of Hb S1c fraction with A2 ⁽⁴⁾
E	falsely elevated		coelution of Hb E with A2 ⁽⁴⁾
D	underestimated		⁽³⁾
G-Philadelphia	falsely elevated		coelution of G-Philadelphia with A2
Lepore	falsely elevated		coelution of Lepore with A2 ⁽³⁾
A1c (elevated)		falsely elevated	⁽³⁾



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<http://www.mlo-online.com/features/201110/clinical-issues/hemoglobinopathy-and-thalassemia-detection.aspx>



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