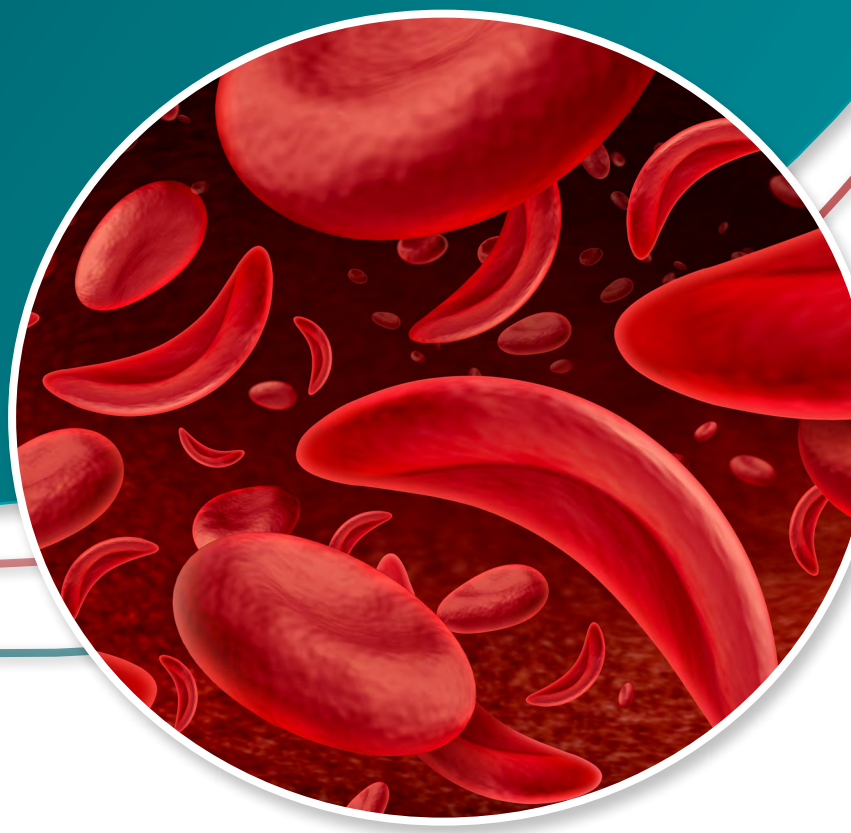
Hemoglobinopathies

Current Practices for Screening, Confirmation and Follow-up



APHL ASSOCIATION OF PUBLIC HEALTH LABORATORIES®

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Executive Summary

Hemoglobinopathies are a group of genetic disorders in which there is abnormal production or structure of the hemoglobin protein.

Sickle cell disease (SCD) is one such blood disorder caused by the abnormal hemoglobin that damages and deforms red blood cells. The abnormal red cells break down causing anemia and obstruct blood vessels leading to recurrent episodes of severe pain and multi-organ ischemic damage. SCD affects millions of people throughout the world and is particularly common among people whose ancestors come from sub-Saharan Africa, regions in the Western Hemisphere (South America, the Caribbean and Central America), Saudi Arabia, India and the Mediterranean, such as Turkey, Greece and Italy. There are an increasing number of people being treated with bone marrow transplants and there are new genetic therapies, though few have access to them.[1] The clinical severity of SCD varies, ranging from mild—sometimes asymptomatic—to severe disease requiring frequent hospitalization. Early access to expert care consisting of education, preventive and disease modifying therapies and SCD screening and treatment of potential complications has led to dramatic improvements in the health and survival of infants and children with SCD. Newborn screening (NBS) for SCD has significantly reduced the burden of the disease and increased survival of affected newborns and children.

Thalassemia is another type of blood disorder that is caused by defects in the genes that produce the globin chains that make up the hemoglobin molecule.[2] There are two main types of thalassemia:

- Alpha thalassemia occurs when a gene or genes related to the alpha-globin protein are missing or changed (mutated) to affect production of the alpha-globin protein. Alpha thalassemias occur most often in persons from Southeast Asia, the Middle East, China and in those of African descent.
- Beta thalassemia occurs when a beta-globin gene is missing or changed (mutated) to affect production of the beta-globin protein. Beta thalassemias occur most often in persons of Mediterranean origin. To a lesser extent, people from China and other parts of Asia and people of African descent are affected.

In 2013, the Association of Public Health Laboratories (APHL) worked with the US Centers for Disease Control and Prevention (CDC) National Center on Birth Defects and Developmental Disabilities, Division of Blood Disorders to convene an APHL Hemoglobinopathy Workgroup to address issues around hemoglobinopathy laboratory testing and follow-up. The workgroup objectives are as follows:

- Identify methods to assist in improving the quality and capability of screening, diagnosis and follow-up for hemoglobinopathies.
- Identify training needs and support standardized training opportunities for public health program staff and executive leadership in the areas of screening, diagnosis and follow-up for hemoglobinopathies.
- Provide technical assistance to international partners related to SCD post-screening (as funding permits).
- Provide better practices for uniformity and reporting of hemoglobinopathies to improve public health data and information.

One of the products of the APHL Hemoglobinopathy Workgroup is this guidance document on hemoglobinopathy laboratory testing and follow-up techniques. The first version of the document was completed in December 2013. This second edition has been updated by the workgroup to explore current screening and diagnostic methods used in laboratories. It also includes an overview of laboratory structure, algorithms for testing, reporting and follow-up from several programs in the US. Furthermore, this document aims to improve and strengthen capabilities by offering advances in current practices, contributing to the goal of early detection of hemoglobin disorders.

Additional resources created and supported by the Hemoglobinopathy Workgroup include:

- Webinars:
  - Approaches to Molecular Testing in Newborn Screening for Hemoglobinopathies
  - Use of Molecular Methods in Hemoglobinopathies Screening: The Clinician Perspective
  - Transfusion Confusion – Identifying Newborns that Have Been Transfused and What to Do About It
  - Alpha Thalassemia: Clinical Aspects
  - Clinical Presentation and Newborn Screening of Beta Thalassemia in the United States
- Publications:
  - Newborn Screening Practices for Beta-Thalassemia in the United States
  - Newborn Screening Practices and Alpha-Thalassemia Detection — United States, 2016 | MMWR

For more information, visit the Hemoglobinopathies Project on the APHL website.

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- Alys Adamski, PhD, MPH
  Division of Blood Disorders and Public Health Genomics, CDC
- Maria del Pilar Aguinaga, PhD, MT (ASCP), MB (ASCP), DLM (ASCP)
  Sickle Cell Center, Meharry Medical College
- MA Bender, MD, PhD
  University of Washington
- M Christine Dorley, PhD
  Maryland Department of Health Laboratories Administration
- Kathryn Hassell, MD
  Colorado Sickle Cell Center, University of Colorado
- Patrick V Hopkins
  Missouri State Public Health Laboratory
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- Rachel Lee, PhD
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Introduction to Hemoglobinopathies

Hemoglobin is a tetramer composed of two alpha-like and two beta-like globin chains (α2β2). Each globin chain carries a heme prosthetic group holding two iron molecules that primarily transport oxygen in the blood.[2,3] Normal adult hemoglobin is designated HbA.[2,3] Variant hemoglobins result from abnormalities of the alpha-globin genes (*HBA1* or *HBA2*) or beta-globin (*HBB*) genes.[2,3,4] More than a thousand hemoglobin variants have been identified, though most have no clinical significance.[3] Qualitative changes due to amino acid alterations result in hemoglobinopathies such as sickle cell or hemoglobin E disease. Quantitative changes due to alterations in regulatory elements, amino acid changes that alter stability, and mutations that alter splicing or processing can alter globin chain production and can contribute to thalassemias.[2,3,4]

Alpha thalassemias are due to a relative deficit in alpha-like chains compared to beta-like chains. The *HBA1* and *HBA2* genes are expressed (four genes in total) in the fetus and after birth. Most commonly, alpha thalassemias are due to alpha-globin gene deletions though some non-deletion mutations exist and tend to be more severe. The number of alpha-globin genes impacted correlates with disease severity.[4] Having one non-functional alpha-globin gene (called silent carrier) has no impact on health but is a factor in genetic counseling. Having two alpha-globin genes impacted (alpha-thalassemia trait) has no significant clinical impact but alters laboratory values and is often confused with iron deficiency. Having three alpha-globin genes impacted (HbH disease) can lead to variable clinical and hematological features and can be severe. Loss of all four alpha-globin genes (Hb Barts Hydrops fetalis) is not typically compatible with life unless treated in utero.[2,4] As such, it is rare for NBS programs to receive these samples. For all alpha thalassemias, identification of potentially impacted family members and genetic counseling are encouraged.

Beta thalassemias are due to a relative deficit in beta-like chains compared to alpha-like chains. Beta-globin variants, deletions or gene regulatory regions can lead to thalassemia as well. Having one deleted or under-producing beta-globin gene (beta-thalassemia trait) is similar to alpha-thalassemia trait with no significant clinical impact but alters clinical laboratory values and is often confused with iron deficiency. Having two deleted or under-producing beta-globin genes can be severe, resulting in “transfusion dependent” or “non-transfusion dependent” thalassemia. Identification of potentially impacted family members and genetic counseling are crucial for all clinically significant beta variants.

Clinically significant variants with altered protein structure are also common and include HbS, HbC, HbD (Punjab/Los Angeles), HbE and O-Arab. A variant in one beta-globin subunit in combination with a normal hemoglobin leads to a carrier or trait status, also known as a heterozygote state. Having a variant in both beta-globin subunits is more likely to result in disease. Having the same variant in both genes results in homozygous disease, for example sickle cell with HbSS. Having two different variants results in a compound heterozygotic disease, for example HbSE, HbSC, HbSD, Hb SO-Arab and HbS beta thalassemia. Regardless of an alpha-globin or beta-globin variant, severity of disease can range from insignificant to serious or life-threatening and early detection has the potential to decrease morbidity, mortality and family stress.[2,3,4,5] Therefore, early detection through NBS is paramount.[6,7]

Hemoglobinopathies, specifically HbSS, HbS beta thalassemia and HbSC disease were added to the Recommended Uniform Screening Panel (RUSP) in 2006. The US Health Resources and Services Administration’s Maternal and Child Health Bureau, in conjunction with the American College of Medical Genetics, determined these disorders to be clinically significant and included them as core targets easily detected by NBS. These were added based on the severity of illness associated with sickle cell disorders.[7] Symptoms can include anemia, severe vaso-occlusive pain episodes and stroke, and can impact virtually every organ system with chronic deterioration over time.[6] Loss of spleen function in infancy dramatically increases risk of invasive infection by encapsulated organisms such as *Streptococcus pneumoniae* (pneumococcus) leading to sepsis and meningitis.

Early detection by NBS followed by confirmatory diagnosis, family education, penicillin prophylaxis and immunizations has dramatically decreased morbidity and mortality in the US. In addition, early diagnosis of SCD prior to the onset of symptoms or complications allows health workers to educate families about SCD and offer anticipatory guidance and disease modifying or curative therapy. These actions—along with screening for the development of complications and connecting families to community-based support groups—are all part of the comprehensive care of the child. Additional hemoglobinopathies readily detected by NBS were also added as secondary targets.[6] Examples include HbE, HbC and HbSE diseases, and both alpha and beta thalassemias.

Methods for NBS and diagnostic testing differ and vary in their sensitivity and the variants detected. While some methods are more automated, others are more manual and labor-intensive. Each method has its advantages and disadvantages, which is why many NBS laboratories utilize more than one method. DNA based methods are being increasingly used.

NBS programs are all structured differently and vary in the number of tiers of testing used and the extent of diagnostic certainty. Some use higher-tiered assays to increase screening specificity on an abnormal result prior to the final NBS result reporting and patient referral for diagnosis of presumptive positive hemoglobinopathies. In addition, reporting and follow-up algorithms differ between programs. Based on these differences, a comprehensive review of methodologies and program structures is warranted.

History of Hemoglobinopathy Screening

Prior to the 1960s, SCD was diagnosed mainly through hematological studies when people presented with clinical manifestations. Many infants were not diagnosed until they presented with severe and life-threatening complications or even death. In the late sixties, only a few states screened newborns for SCD, and then only a subset of newborns. In 1971, in response to pressure by African American advocacy groups, the US government made SCD a scientific and healthcare priority by allocating funds for treatment and research and in 1972 the US Congress signed into law the National Sickle Cell Disease—Sickle Cell Anemia Control Act (Public Law 92-294).[8] Several Sickle Cell Centers were created in the US, mainly associated with universities or medical centers, including some at medical schools of Historically Black College and Universities.[8] A 1973 survey of annual workloads of US state public health laboratories, known as *The Consolidated Annual Report*, showed that 12 state public health laboratories had some form of sickle cell screening program.[9] In 1975, the first universal NBS program for SCD was implemented in New York on a pilot basis.[10] Subsequently, screening was adopted by the remaining states throughout the late 1980s and 1990s and into the 2000s. Initially, screening was targeted for populations at risk—primarily African Americans. However, the missed cases rate was higher than 30% due to difficulty in identifying an infant's race or ethnicity at birth and so universal screening was implemented.[11] Later on, other hemoglobinopathies were acknowledged as important public health issues.

A high mortality and morbidity from encapsulated organisms in children with SCD were noted in the 1970's. About 20% of children with SCD died from these infections before age six. To address this, in 1986 penicillin prophylaxis and improved access to family education were promoted for infants with SCD.[12] Penicillin prophylaxis, family education and improved access to healthcare significantly reduced infection-related mortality and became a powerful incentive to implement widespread neonatal screening for SCD.[12,13,14,15]

Since May 1, 2006, all 50 states and the District of Columbia have required and provided universal NBS for SCD. Notably this was over a quarter of century after the national recommendation in 1987.[9,12] NBS is well recognized as the largest and most successful health promotion and disease prevention system in the US. NBS is the practice of testing every newborn for certain harmful or potentially fatal conditions that are not otherwise apparent at birth. A blood sample is collected before the newborn leaves the hospital or birthing center and identifies serious, life-threatening conditions before symptoms begin, giving a chance to reduce potential harm. Although such conditions can be rare, they can affect a newborn's normal physical and mental development. Early detection is crucial in NBS since intervention can prevent death or a lifetime of severe disabilities.

Along with the initial NBS goal to identify SCD, other hemoglobin disorders such as, alpha and beta thalassemia have gained significant attention in recent years due to the rapidly changing demographics and awareness in the US as a result of increased immigration. Hence, hemoglobin disorders common in other geographic areas are being seen more frequently in the US.[16]

Originally, laboratories used citrate agar electrophoresis for screening of abnormal hemoglobins in cord blood. Improvement in hemoglobinopathy electrophoretic techniques have made it possible to screen newborns using either cord blood or heel stick samples (dried blood spots, or DBS).

Currently, hemoglobinopathy screening programs use a combination of isoelectric focusing (IEF) and/or high-performance liquid chromatography (HPLC) for either primary or secondary screening methods (**Table 1**). Some use a third complimentary technique such as a DNA-based assay to confirm specimens with abnormal screening results.[13] See **Table 1** for a distribution of laboratories versus methods utilized. The current screening methodologies are sensitive and specific in detecting high risk infants; however, each method has its own unique benefits and limitations.

Table 1: Number of Laboratories Participating in CDC's NBS Quality Assurance Program (NSQAP)*

Primary Method	Secondary Method	Number of Laboratories	
		US*	International
HPLC (High Performance Liquid Chromatography)	**None**	19	16
HPLC	**IEF** (Isoelectric Focusing)	10	•
HPLC	**Capillary Electrophoresis**	0	•
HPLC	**Cellulose Acetate**	0	•
HPLC	**HPLC**	•	•
IEF	**None**	4	•
IEF	**HPLC**	9	•
IEF	**Real Time PCR** (Polymerase Chain Reaction)	•	0
Capillary Electrophoresis	**None**	0	•
FIA-MS/MS (Flow Injection Analysis Tandem Mass Spectrometry)	**None**	0	•
FIA-MS/MS	**HPLC**	0	•
Real Time PCR	**FIA-MS/MS**	0	•

Types of Specimens for Hemoglobinopathy Screening and Diagnosis

At one time, programs in the US required cord blood for hemoglobinopathy screening; however, programs now use DBS, which are collected from a heel prick and spotted onto filter paper.[17] For additional information on NBS specimen collection, refer to the Clinical and Laboratory Standards Institute's (CLSI) document *NBS01-7th Ed, Blood Collection on Filter Paper for Newborn Screening Programs*.[18] NBS specimens should be properly collected to avoid clotting, smearing, inadequately filled circles, oversaturation or scratching by capillary tube. Although these specimens may be unsatisfactory for some NBS tests, they may be acceptable for hemoglobinopathy testing.[18] States vary on how they handle, test and report unsatisfactory/poor quality NBS specimens.

DBS or liquid whole blood can be used for screening and/or confirmation of children over one year of age and adults. There are changes in hemoglobin types and quantities through the first year of life, but after the first year an individual's hemoglobin types tend to remain the same.

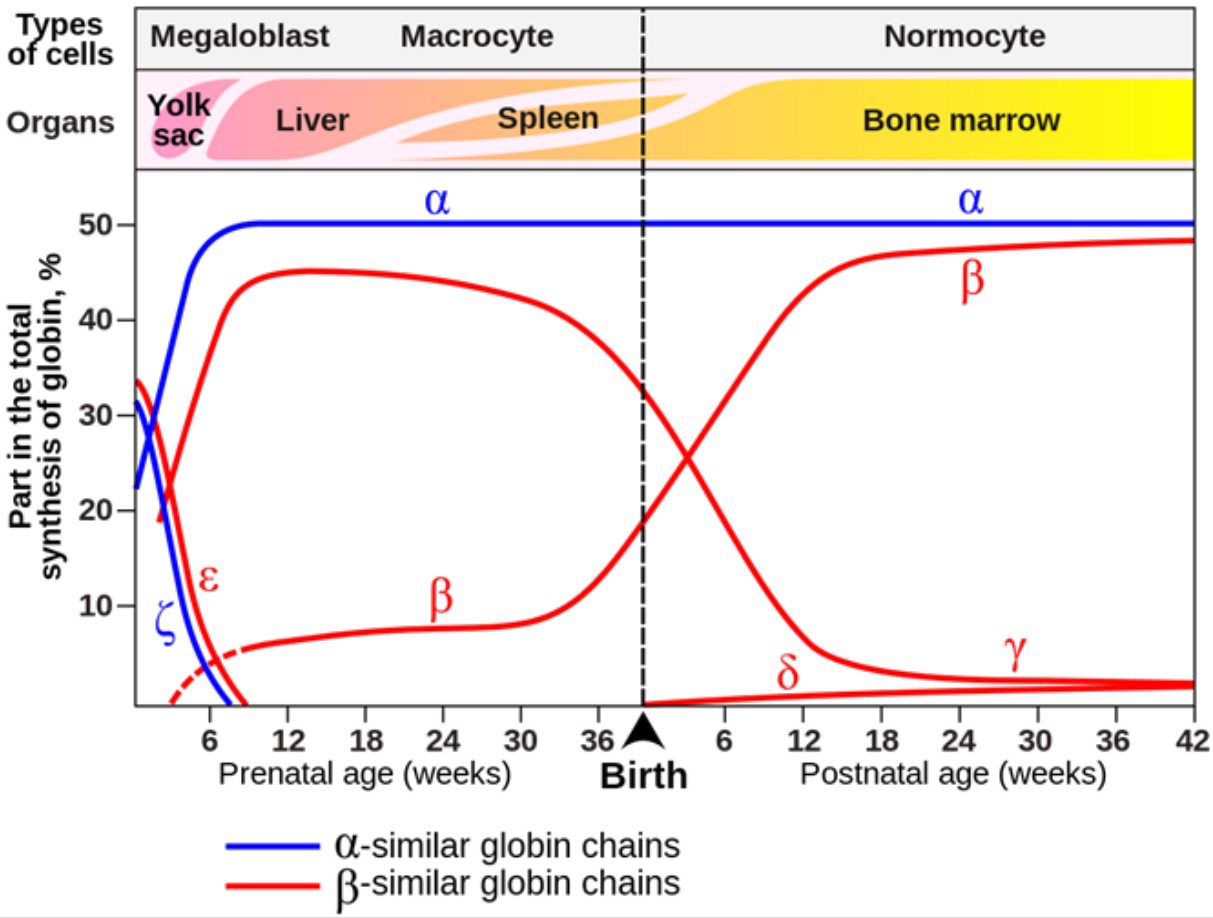
Figure 1 shows changes in relative proportions of globin chains at various stages of embryonic, fetal and post-natal life.[19]

* Source: NSQAP: About Newborn Screening Laboratories (accessed April 1, 2025).

* Represents all US and additional non-US programs

• Three or fewer laboratories use this testing scheme.

Figure 1: Globin switch during in utero and post-natal life*



For the collection of whole blood samples, Ethylene Diamine Tetra Acetic Acid (EDTA) is the typical anti-coagulant used. Heparin may interfere with DNA amplification by polymerase chain reaction (PCR). For DBS collected from a finger stick, the palmar surface of the finger's last phalanx is most frequently used, see CLSI Collection of Capillary Blood Specimens document GP42Ed7 for additional information.[20] High heat and humidity can change the levels of hemoglobin A and S in DBS samples.[21] To maintain the integrity of hemoglobin proteins, high humidity and temperature should be minimized during transport and storage. However, DBS collection from a finger stick is not recommended for newborns.

Blood smears should not be used for hemoglobinopathy screening. A smear is unreliable as it requires the presence of sickled cells which may or may not be in circulation at the time of collection. Blood smears also cannot differentiate homozygous from heterozygous traits, nor can they detect other hemoglobin variants.[22]

Transfusion may affect hemoglobinopathy screening results. The transfused blood can mask a hemoglobinopathy, or the transfused blood may contain a hemoglobin variant that does not belong to the patient. In cases of transfusion, hemoglobinopathy screening should be repeated four months post transfusion unless DNA testing is conducted according to the CLSI *Newborn Screening for Hemoglobinopathies NBS08*,[23] or according to individual program guidelines and the extent of transfusion.

There are several different methods as next described that are used for hemoglobinopathy screening and confirmation. Specific specimen requirements may vary between these methods.

* Source: Postnatal_genetics.svg licensed with Cc-by-sa-3.0, GFDL

Methodologies for Hemoglobinopathy Screening and Diagnosis

Various methods for hemoglobinopathy NBS and adult testing are employed by US laboratories, ranging from traditional hands-on methods, to automated high-throughput methods, to molecular assays.

Many programs have historically used electrophoresis as the method of choice for identification and quantification of variant hemoglobins.[24] In more recent decades, with the introduction of universal screening of newborns, technology has emerged that is more robust, with high-throughput and greater sensitivity over electrophoresis.[24,25] Many of the same methods used for screening, such as IEF and HPLC, are also used for diagnosis. Laboratories employ one or more methods in their protocol for detection of hemoglobinopathies. Choice of method depends on the laboratory and its testing algorithm.

More recently, molecular methods have emerged and are being utilized by some screening and diagnostic laboratories to obtain a more comprehensive analysis and determine the nature of the hemoglobinopathy and its clinical implications. Some advantages to molecular testing include differentiation of disease from trait in cases where the infant received a transfusion, improved timeliness to diagnosis and providing universal testing access.

Below are descriptions of methods used to screen hemoglobinopathies.

Isoelectric Focusing

IEF separates proteins in a gel medium that has a pH gradient consisting of ampholytes (zwitterions). When a high voltage is applied, narrow buffered zones are created with stable, but slightly different pHs (**Figure 2**). Slower moving proteins migrate through these zones and stop at their individual isoelectric points (pI).[3] In the case of hemoglobin, these migrate to a zone in the medium where the pH of the gel matches the hemoglobin's pI. At the pI, the net charge of the hemoglobin becomes zero and ceases to migrate. The hemoglobin migration order of IEF is similar to alkaline electrophoresis. Resolution is clear with differentiation of HbC from HbE and HbO and HbS from HbD and HbG, respectively. HbA and HbF are also clearly differentiated.[24] With neonatal specimens, a distinct band representing acetylated HbF is readily identified and slightly anodic to HbA.[26] Staining may be necessary depending on the manufacturer's recommendations (**Figure 3**). Gels may be read wet or dry and band identification is accomplished by comparison to migration patterns of known quality controls.[26] This method allows for greater precision and accurate quantification than standard electrophoresis. It gives excellent resolution in addition to high-throughput, but is also more manual and time consuming.[3, 24, 26] An alternative method is needed to confirm or differentiate hemoglobin variants (bands other than HbF or HbA).[26] Fast migrating bands (Hb Barts, HbH) can also be identified by IEF.

Figure 2. Isoelectric Focusing

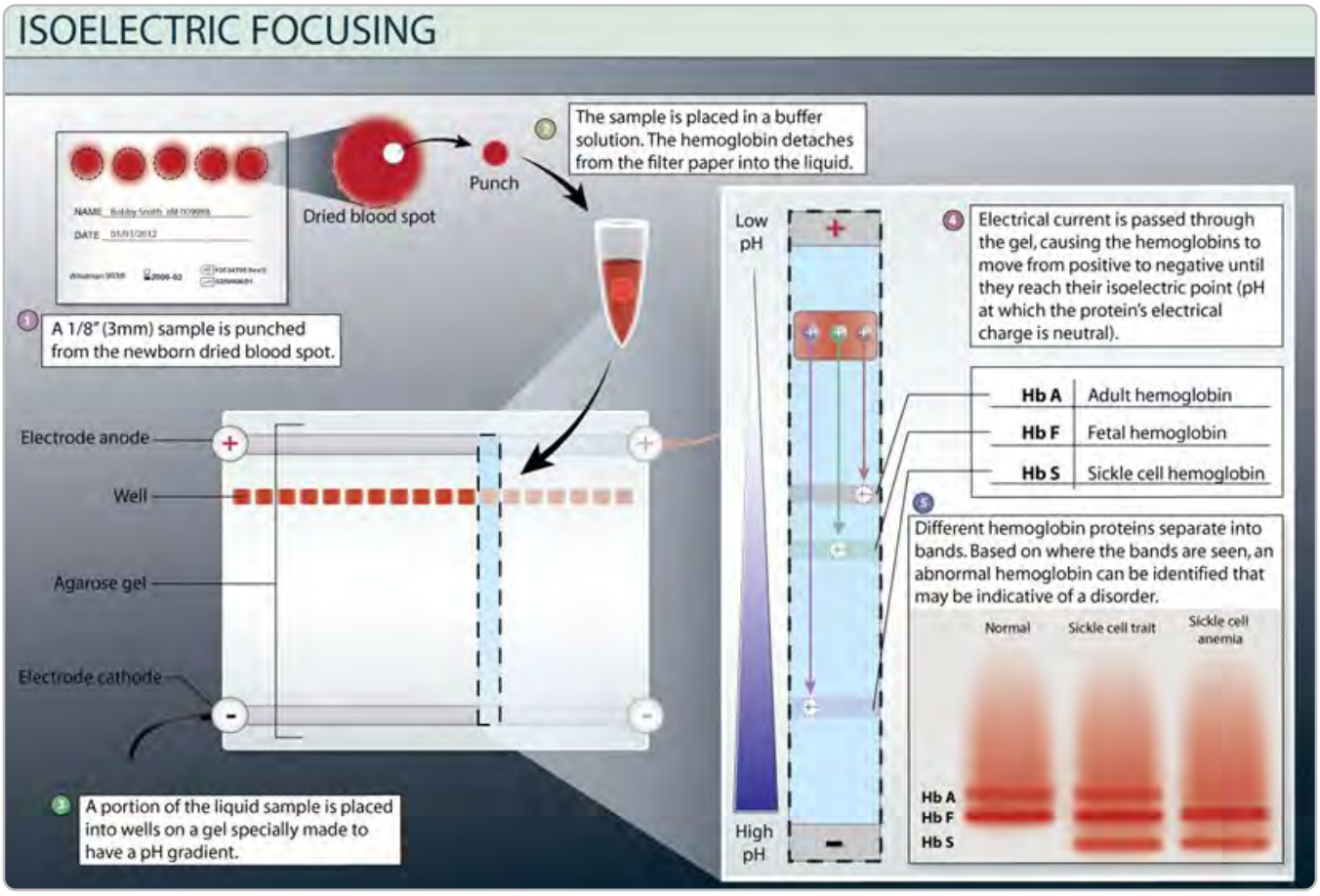
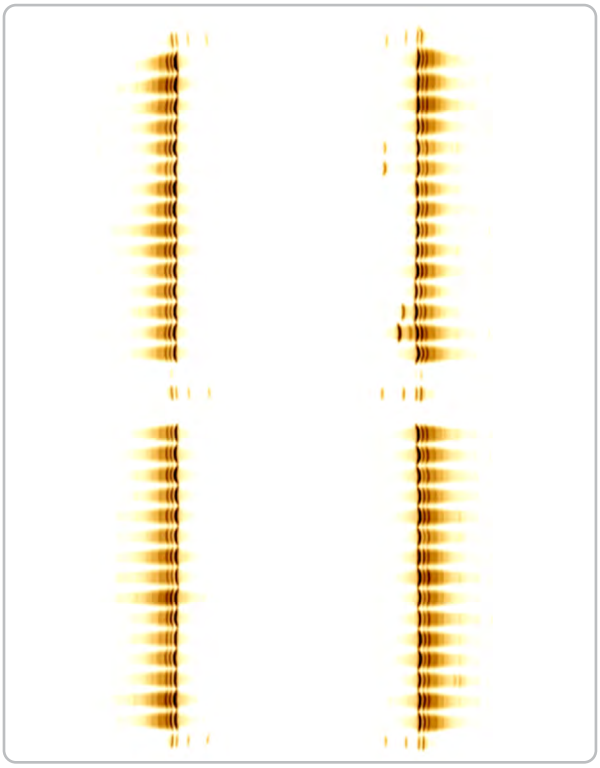


Figure 3. Stained and Dried IEF Gel

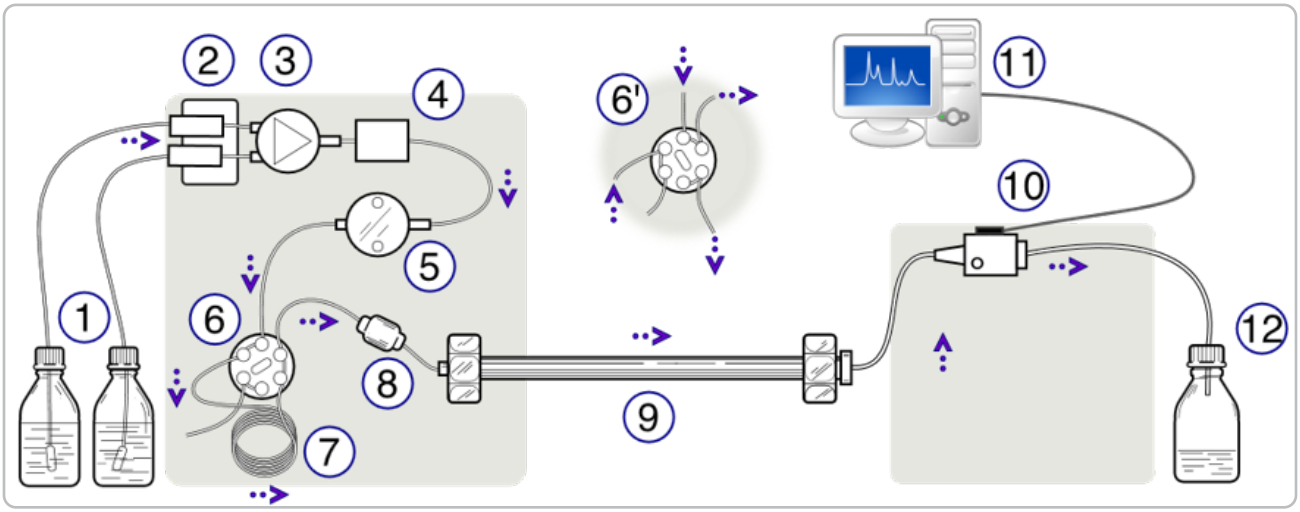


High Performance Liquid Chromatography

Hemoglobins are separated using cation exchange HPLC with an analytical cartridge and a preprogrammed buffer gradient that increases in ionic strength over time (**Figure 4**). The hemoglobin fractions separate based on their ionic interaction with the cartridge.[26] The separated fractions pass through a flow cell, where absorbance is measured at 415 nm and again at 690 nm to reduce background noise. Changes in absorbance are monitored over time, producing a chromatogram (absorbance vs. time). Each hemoglobin has its own characteristic retention time and is measured from the time of sample injection into the HPLC to the maximum point of each peak. Identification of unknown hemoglobin is achieved through comparison with known hemoglobin retention times.[27] If a peak elutes at a retention time not predetermined, it is labeled as an unknown.

HPLC achieves good separation and quantitation of HbF and HbA2, in addition to screening for variant hemoglobins along with thalassemia. HPLC is highly reproducible, offers simplicity with automation, rapid results and quantitation.[27]

Figure 4. High-performance Liquid Chromatograph



Schematic representation of an HPLC unit. (1) Solvent reservoirs, (2) Solvent degasser, (3) Gradient valve, (4) Mixing vessel for delivery of the mobile phase, (5) High-pressure pump, (6) Switching valve in "inject position", (6') Switching valve in "load position", (7) Sample injection loop, (8) Pre-column (guard column), (9) Analytical column, (10) Detector (i.e. IR, UV), (11) Data acquisition, (12) Waste or fraction collector. "HPLC apparatus" by WYassineMrabetTalk.

This vector image was created with Inkscape. - Own work. Legend based on: Practical High-performance Liquid Chromatography by Veronika Meyer, 4th edition, John Wiley and Sons, 2004, ISBN 0470093781, p. 7 and chromatography-online.org. Used file: Computer n screen.svg (Crystal SVG icons). Licensed under CC BY-SA 3.0 via Wikimedia Commons.

Cellulose Acetate Electrophoresis (Alkaline)

Cellulose acetate electrophoresis or alkaline electrophoresis makes use of the negative charge which hemoglobin will adopt under alkaline conditions. Samples are applied to cellulose acetate agar gel and hemoglobins are separated by electrophoresis using an alkaline buffer (Tris-EDTA with Boric Acid) at pH 8.[4, 28] Each hemoglobin variant carries a different net charge so it will migrate at varying speeds. Following electrophoretic migration, visualization of hemoglobin bands are accomplished through staining with Ponceau S, Amino Black and Acid Violet or other similar stains and compared with known standards.[3, 26, 28] Hemoglobins are quantified using densitometric scanning and the relative percentage of each band is determined.[26, 28]

This method yields rapid and reproducible separation of HbA, HbF, HbS and HbC as well as other variant hemoglobins with minimal preparation time. However, due to limited sensitivity and because some hemoglobins are electrophoretically similar though structurally different, an alternative procedure must be incorporated into the screening algorithm for differentiation of these hemoglobins. For example, HbS, HbD, HbG and Hb Lepore co-migrate, so they are indistinguishable on alkaline electrophoresis. The same is true for HbC, HbA2, HbO-Arab and HbE.[13, 24, 28]

Citrate Agar Electrophoresis (Acid)

Electrophoresis occurs in an acidic environment at pH 6.[2, 28] This method is based on the complex interactions of the hemoglobin with the electrophoretic buffer and the agar support. Staining allows visualization of hemoglobin bands. Acid electrophoresis allows confirmation of variant hemoglobins observed in the cellulose acetate electrophoresis procedure and allows good separation of HbC from HbE and HbO-Arab.[13] It permits additional separation of HbS from HbD and HbG.[23, 28] Citrate Agar electrophoresis appears to be more sensitive than cellulose acetate for detecting HbF.

Alkaline Globin Chain Electrophoresis

The hemoglobin molecule can be separated into its globin chain components and heme groups by the addition of 2-mercaptoethanol and urea. At alkali pH, these globin chains will migrate to their characteristic position on a cellulose acetate strip, under an electrical field. The characteristic migration pattern aids in the identification of each globin chain type.[29]

Capillary Zone Electrophoresis

Charged molecules are separated by their electrophoretic mobility in an alkaline buffer (pH 9.4) with separation occurring according to the electrolyte pH and endosmosis or electroosmotic flow.[25] Capillaries function in parallel allowing eight simultaneous analyses. Samples are hemolyzed and injected into the anodic end of the capillary. High voltage protein separation is performed and hemoglobins migrate from the anodic end of the capillary appearing in specific zones to the cathodic end where detection occurs at 415 nm. Results are assessed visually for abnormalities with identification of normal and disease patterns.

There is improved detection of SCD due to separation of hemoglobin fractions which enables differentiation of HbS from other variants. Capillary zone electrophoresis allows clean separation of HbE from HbA2 and facilitates easier detection of HbBarts and HbH.[25]

Figure 5. Principle of Hemoglobin Electrophoresis Using Capillary Electrophoresis Technologies

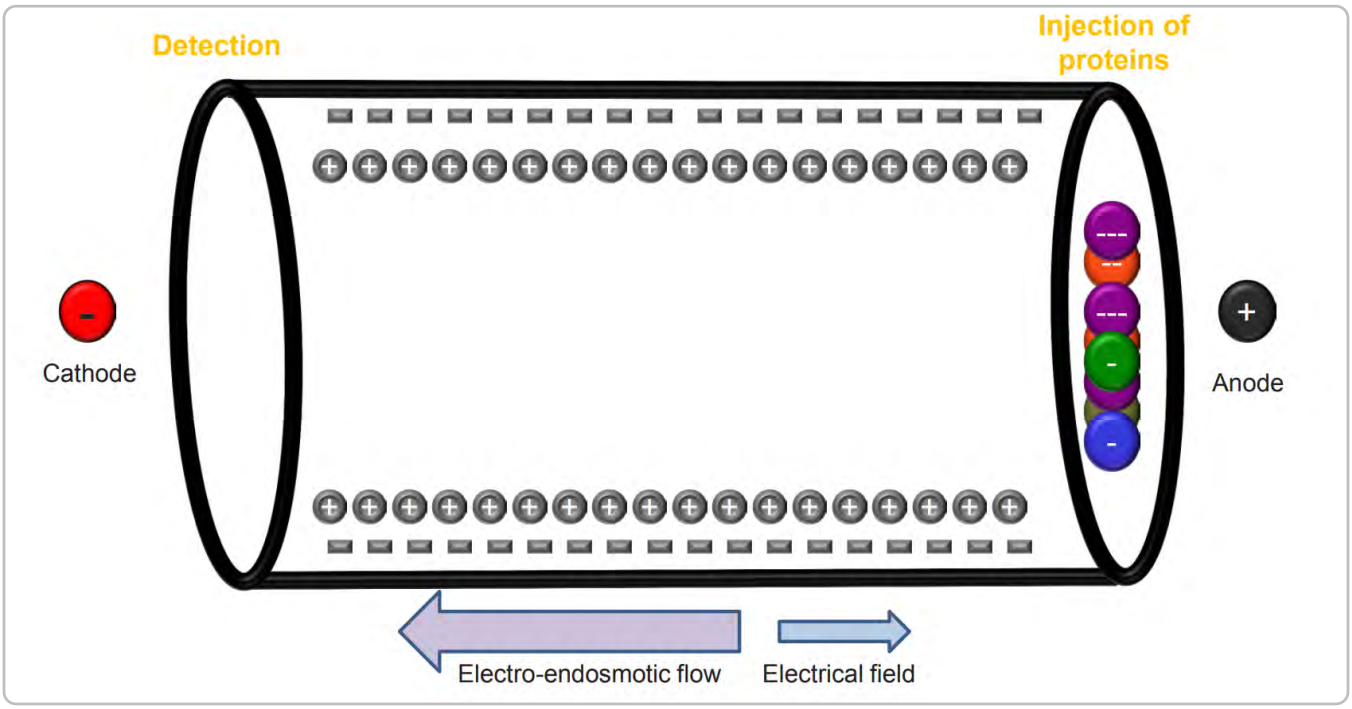


Figure 6. Capillaries Diagram

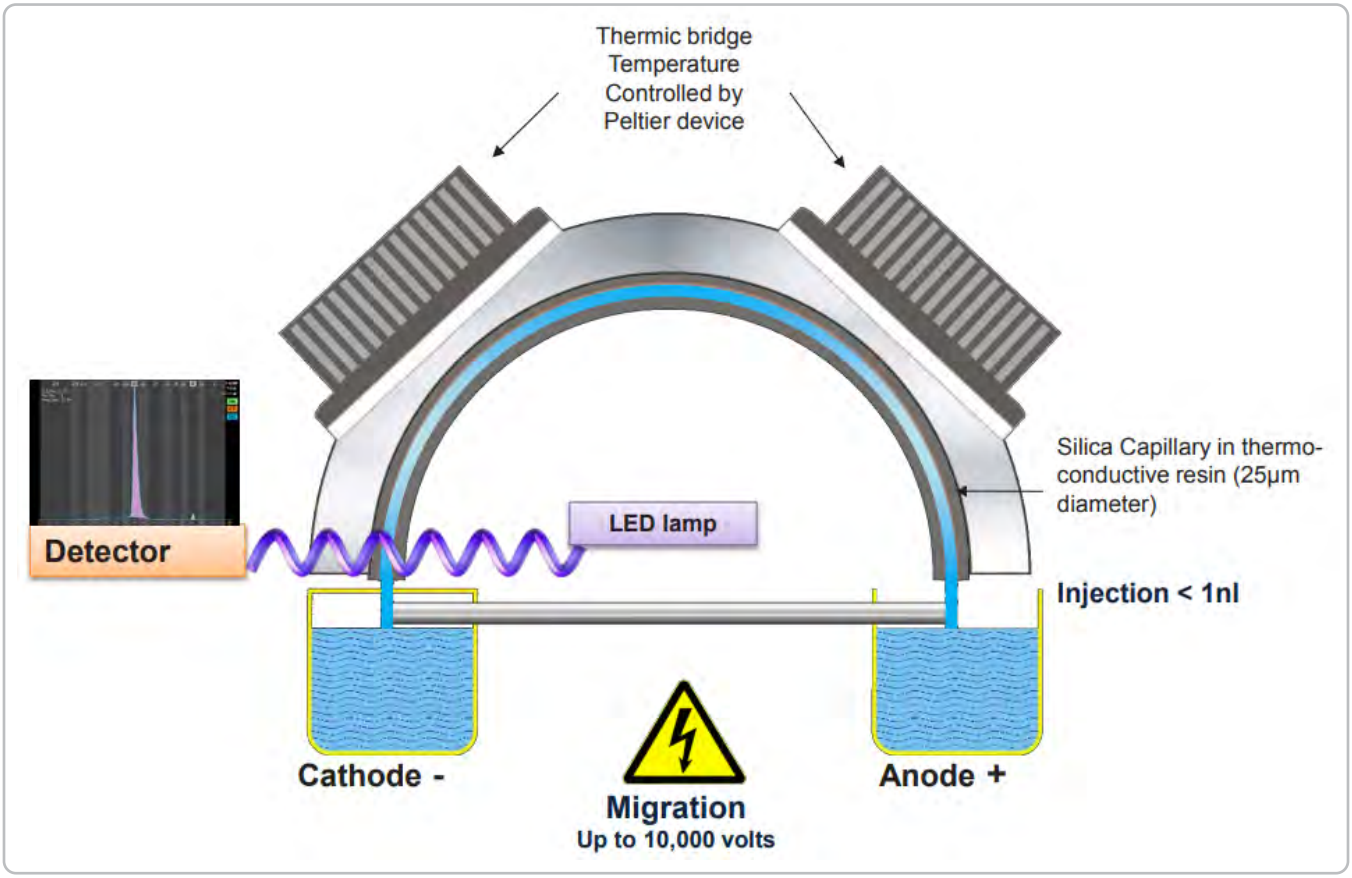
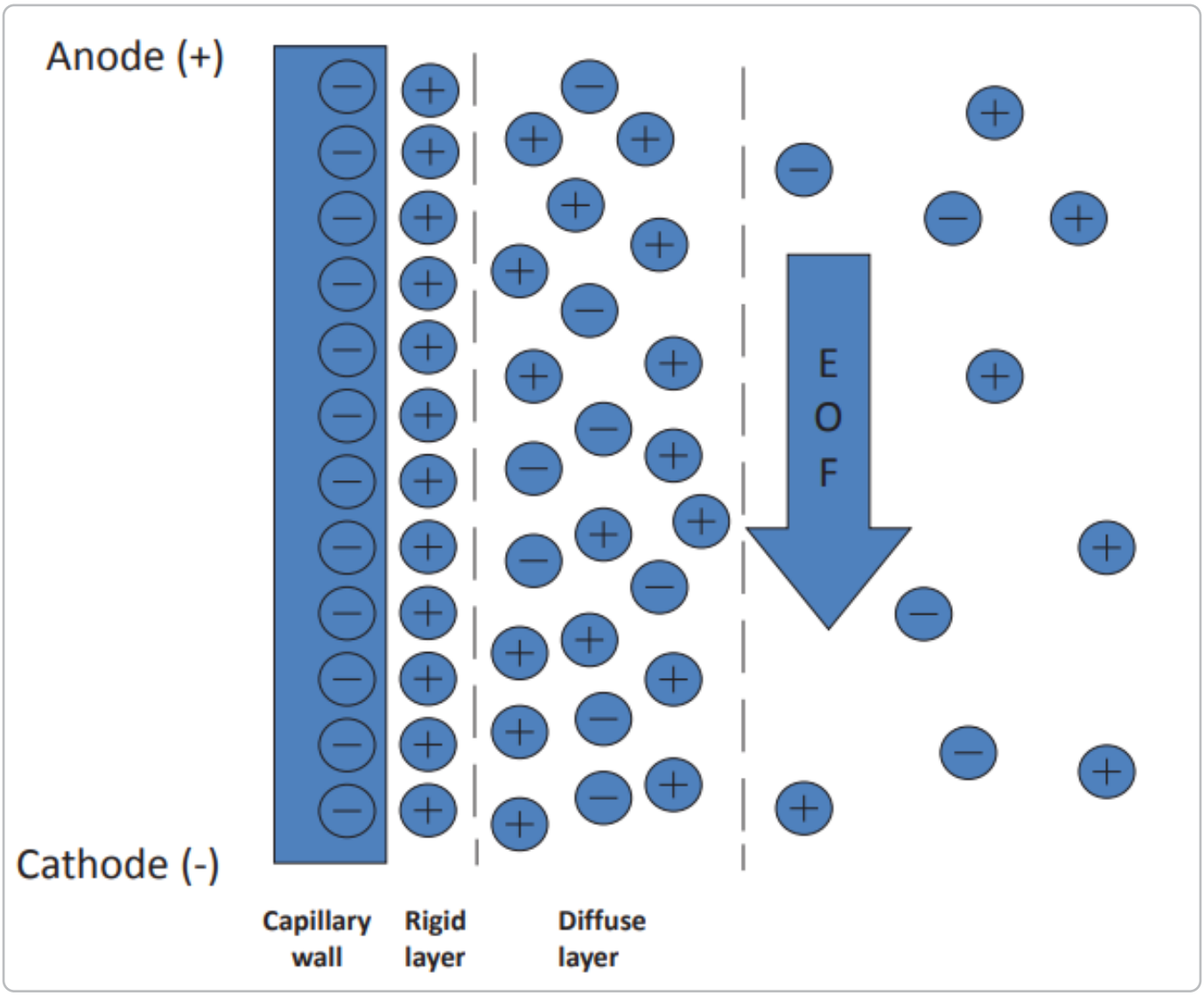


Figure 7. Net Flow of Buffer towards the Negative Electrode (Cathode)



Molecular Methods

Molecular testing for hemoglobinopathies is expanding rapidly in both its use and the technologies utilized. There is Restriction Fragment Length Polymorphism (RFLP), Allelic Discrimination using Real Time PCR end-point data, PCR-based deletion analysis and DNA Sequencing. DNA extraction of blood from the DBS filter paper matrix is needed for all PCR-based assays. Methods for DNA extraction include crude boiling preparations, alkaline lysis preparations and commercial methods. Each one has its own benefits and limitations, and each laboratory needs to determine the best method for their applications.[30]

Considerations for all PCR-based Assays

All molecular assays using PCR are susceptible to contamination by aerosolized amplicons. To minimize the risk of contamination, at a minimum, assay set-up and amplification/analysis must be separated and a one-way directional workflow is required.[31] Depending upon the volume of specimens tested and nature of the work, laboratories may require shoe covers and hair caps in addition to a laboratory coat and gloves.

Each PCR run must include a positive control (a genotype positive DBS or genomic DNA, i.e., SS, SC, AS, AC, etc.) and a negative control, also referred to as a no template control (no DNA control i.e., water or reagent only). The positive control verifies that the amplification occurred, and the negative control will detect contamination of reagents.

Allelic Discrimination Using Real-time PCR

Allelic Discrimination (AD) measures fluorescence of a sequence specific probe to determine if a variant is present. For this technique, a forward and reverse primer spanning the area of interest are used as well as hydrolysis probes.[32,33] Hydrolysis probes are oligonucleotides that have a fluorophore bound to the five prime (5') end and a quencher molecule bound to the three prime (3') end. When the quencher is in close proximity to the fluorophore, fluorescence cannot be detected. After DNA is extracted from the specimen, a PCR reaction with the forward and reverse PCR primers, DNA polymerase, nucleotide triphosphates, (dNTPs) and two probes is prepared. One probe corresponds to the normal sequence and the other corresponds to the mutant sequence. The fluorophore bound to each primer emits fluorescence at different wavelengths and can be distinguished. During PCR, the primers and probes bind to their complementary sequences. When DNA polymerase encounters the bound probe, the polymerase's 5'–3' exonuclease activity degrades it. The fluorescent molecule is released, separating it from the quencher and the fluorescence can be measured. Because amplification occurs exponentially, there is a large increase of fluorophores that are no longer in proximity to the quenchers. The fluorescence is measured in a real-time PCR machine and the results are analyzed by the software. If the signal from only one probe is detected, the sample is homozygous for the sequence complementary to that specific probe, or the other allele has been deleted. If the signals from both probes are detected, then the sample has both a mutant allele and a normal allele and is considered a carrier.

This method has a higher throughput than RFLP because the results are generated right after the amplification ends, with no further processing. All reactions take place in the PCR tube which never has to be opened, thus reducing the risk of contamination. The throughput can be increased by using 96- or 364-well PCR plates and automation. Limitations include an increased cost for the probes and the need for a real-time PCR machine.

DNA Sequencing

Sanger sequencing determines the exact sequence spanning the area of the primers used. Once DNA is extracted from the sample, it is amplified by PCR but in addition to the primers, the DNA polymerase and dNTPs, dideoxynucleotides (ddNTPs) are added. Each of the four ddNTPs has a specific fluorophore bound to it, which emits a signal at a different wavelength. During amplification, when one of the ddNTPs is incorporated, extension stops because the lack of a hydroxyl group does not allow for the addition of the next base. The ratio of dNTPs to ddNTPs is optimized to generate fragments that are one base different than the next. After amplification, the products are purified, typically by DNA precipitation or by spin column.

Next, they are loaded onto a capillary electrophoresis instrument that separates the products by size. When the products move across the path of a laser in the instrument, each fluorophore emits a signal at a different wavelength and this information is captured, analyzed by the instrument software and the sequence is displayed (**Appendix A**, **Figure A4**).[34]

The greatest benefit of this method is that every base change in the area of interest is determined including single nucleotide polymorphisms, small insertions and small deletions. Sequencing is a high-throughput method, but all the sequence data must be reviewed by a trained technician, which can take a large amount of time depending on the length of sequence and the number of samples. For NBS laboratories, if sequencing is used for hemoglobinopathy screening, only the beta-globin gene, *HBB*, is usually sequenced because it is relatively small, and the most common variants tested are point mutations in *HBB*. The alpha-globin genes, *HBA1* and *HBA2*, have greater than 96% sequence homology making sequencing of these genes more complicated. Many of the common alpha-thalassemia variants are large deletions which cannot be found by sequencing.

Programs use different approaches for hemoglobinopathy screening, confirmation and diagnosis. NBS programs do not routinely screen for the purpose of detecting alpha or beta thalassemias, although screening methods may detect some forms of these hemoglobinopathies. The clinical presentation, along with hematologic features observed in the complete blood count (CBC) and peripheral blood smear, as well as molecular studies, contribute to the confirmation and diagnosis of thalassemias. Family studies are warranted along with genetic counseling.[24]

To further improve screening and diagnosis of hemoglobinopathies including thalassemias, some laboratories are exploring the use of tandem mass spectrometry and whole genome sequencing. These methods are promising improved sensitivity and specificity for hemoglobinopathies. However, as procedures advance, others become less ideal for screening. For example, HbS solubility tests, although simple and easy to use, often cannot distinguish disease and trait and are characterized by false positives from other hemoglobin types and interfering substances that may be present in the sample. False negatives are commonly seen in patients with low hemoglobin or hematocrit levels, profound anemia or high fetal hemoglobin. Other abnormal hemoglobins are not readily detected and result interpretation can be difficult due to the subjectivity of the test.[25] Based on these limitations, solubility testing is not recommended.

Additionally, when deciding on which method is most appropriate for hemoglobinopathy detection, it is necessary to consider the benefits the method will bring to the individual program. Likewise, it is important to consider expense, throughput, ease of use and skills required for implementation and routine analysis. Methods also have different limitations that must be evaluated. For example, peak or band resolution, differentiation of hemoglobins and result interpretation are important limitations to evaluate. The next section explores some of the limitations of the more commonly used applications.

Method Advantages, Limitations and Testing Strategy

As of 2023, the majority of hemoglobinopathy screening programs use a combination of IEF and HPLC for either primary or secondary screening methods (**Table 1, page 9**). Both have advantages and limitations. The optimal strategy is to use a method that fits the laboratory the best as a first-tier test and use a complementary technique as a second-tier test to increase the specificity of screening. **Figures 8–15** show various patterns using different methods.

Isoelectric Focusing

Advantages

- Allows the reader to view as many as 80 hemoglobin profiles per gel. This is very beneficial in high-volume laboratories.
- Variant bands are easily distinguished on a gel when compared alongside normal Hb FA profiles and appropriate controls.

Disadvantages

- Hemoglobin bands can leak into neighboring profiles, be poorly focused, distorted or contaminated creating subjective interpretations.
- This can be overcome by having multiple analysts enter results followed by an experienced analyst who makes the final call.
- Difficult to quantitate hemoglobin bands when reading IEF patterns. Quantitating hemoglobin bands is either subjective, requires a visual inspection or a densitometer.
- Gel preparations are manual, requiring multiple steps in the process.

Figure 10 is a good example of both alpha thalassemia trait (FAB2) and Hb S trait. FAS is also known as S trait due to the fact that it is a heterozygote (only one copy of chromosome 11 has the variant for βS). In a homozygous S, there is no A. Homozygous S is also called SCD and the phenotype code is HbFS.

Figure 8. Normal Hemoglobin (HbAA) by IEF with age

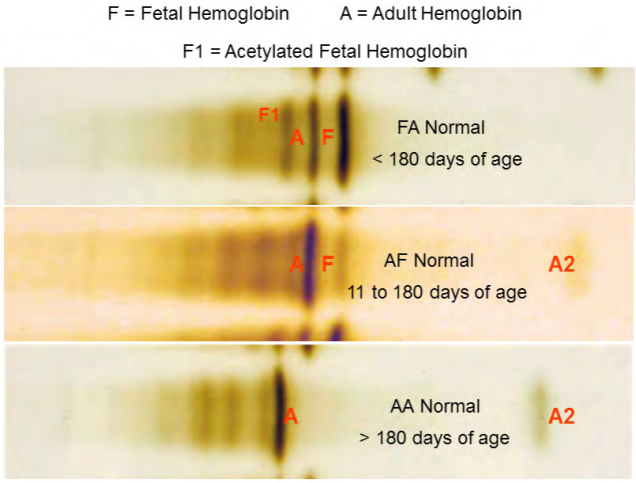


Figure 9. Sickle Cell Disease (HbSS) by IEF

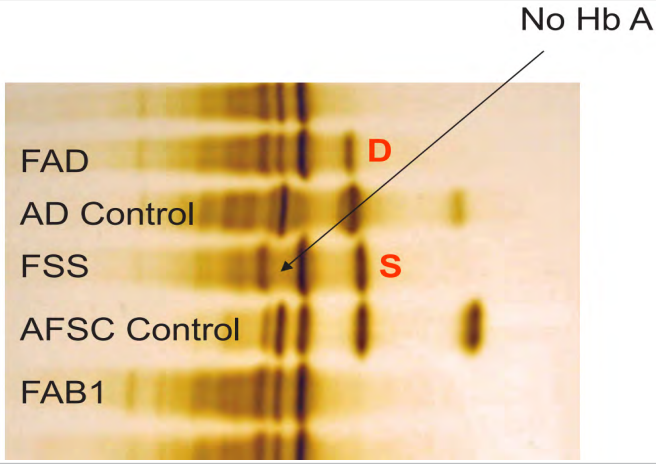
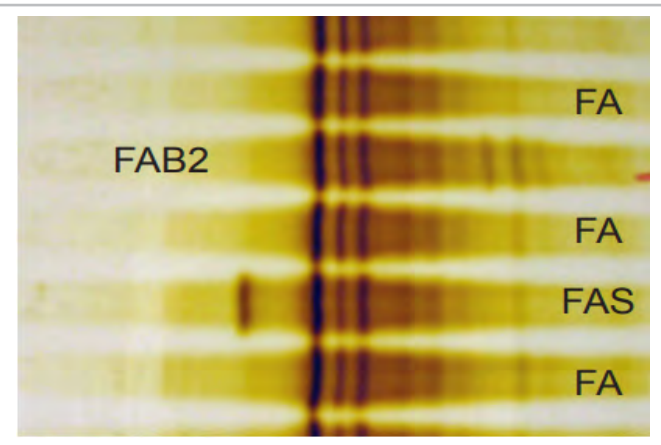


Figure 10. Sickle Cell Trait (Hb AS) and Alpha Thalassemia Trait (FAB2) by IEF



High Performance Liquid Chromatography

Advantages

- Typically includes software that calculates the percent area of each hemoglobin peak. This helps with the determination of thalassemias and phenotype reporting.
- Automation decreases hands on time.
- Peaks within retention time windows can be identified by software algorithms, which also assists the analyst in making the final determination.

Disadvantages

- Software determination should not be used to assign final phenotype results without examining each chromatogram.
- HPLC resolution can be low in some systems causing variant bands to be overlooked making manual review imperative.
- A known issue with primary HPLC screening is system plugging due to filter paper fibers from the blood spot.

Figure 11. Sickle Cell Disease (HbSS) by HPLC

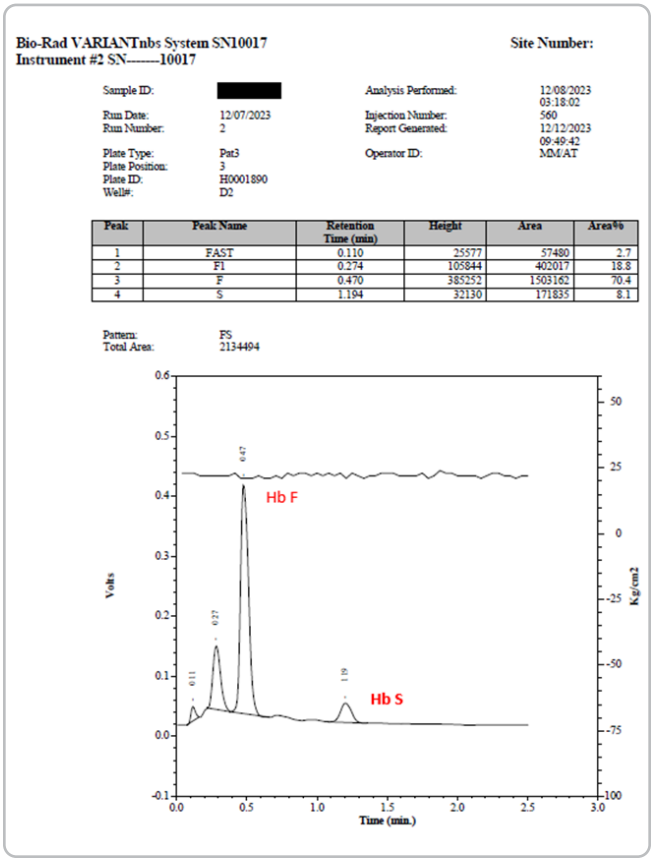
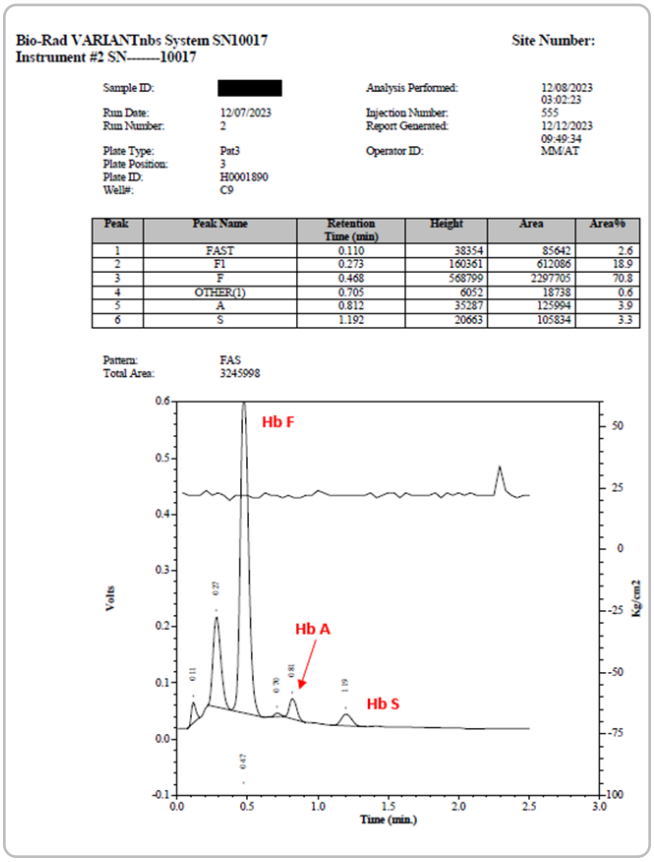


Figure 12. Sickle Cell Trait by HPLC



Capillary Zone Electrophoresis

Advantages

- Capillary zone electrophoresis (CE) is commonly used in diagnostic laboratories and is becoming an excellent choice for high volume laboratories.
- Automation decreases hands on time.
- This method better separates HbA2 from HbE, HbC, Hb Lepore, and HbS than the HPLC method.[35]

Disadvantages

- Due to resolution and sensitivity, however, not all hemoglobin variants are detectable by this method.
- Similar to HPLC, this system also has problems with filter paper plugging.
- HB Barts is the slowest fraction to migrate to the detector on capillary zone electrophoresis (**Figure 15**), unlike HPLC where HB Barts is the fastest migrating fraction.

Figure 13. *FAS* Pattern on CE

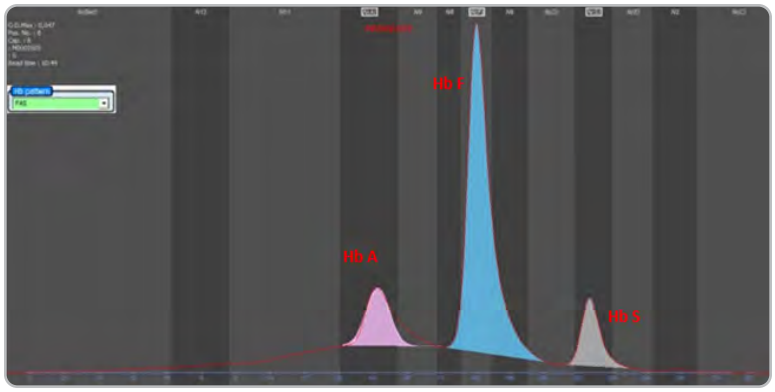
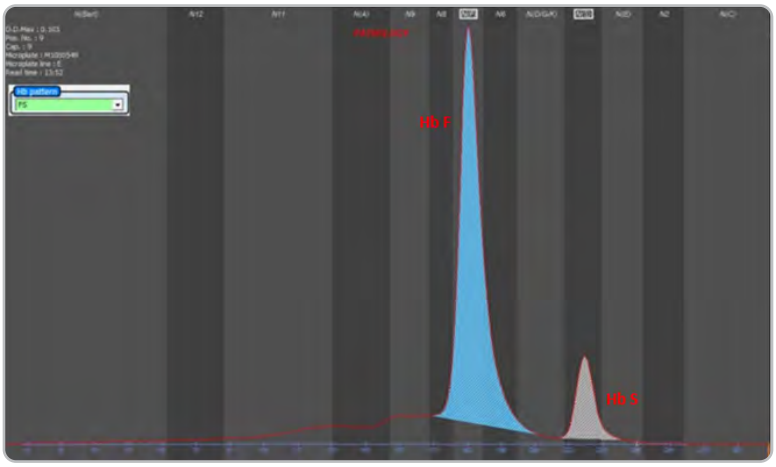


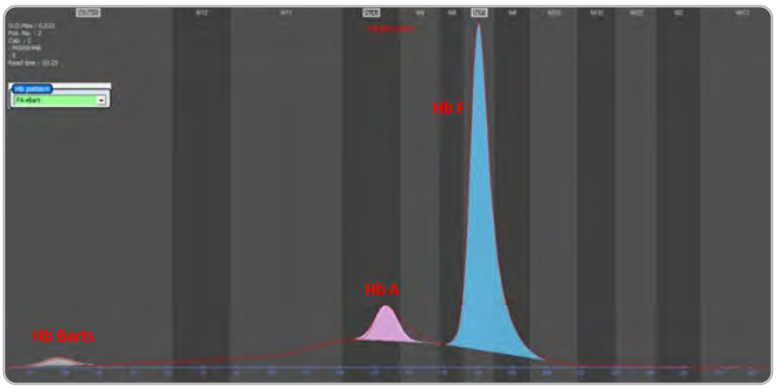
Figure 14. *FS* Pattern on CE



Other Methods

Less commonly, citrate and cellulose acetate are being used by some laboratories. These methods have limited sensitivity and require alternative methods for differentiating abnormal hemoglobins sharing the same or similar electrophoretic mobility. Interpretations can be subjective.

Figure 15. *Barts Hemoglobin* Pattern on CE



Molecular testing can be added to resolve cases when the newborn has been transfused with packed red blood cells. Since the newborn's phenotype is masked by the donor, DNA testing can be used to identify any abnormal hemoglobins. This method is limited by workspace issues required for unidirectional flow, training and relatively high cost. Also, it is difficult to obtain proficiency and quality control materials which are required for certification through accreditation bodies.

Despite limitations and advantages, programs must consider how to effectively implement testing protocols for optimal workflow and efficiency for hemoglobinopathy detection. Specific examples of algorithms for hemoglobinopathy detection are provided in the Appendix. All algorithms presented, despite their corresponding program, aid in meeting the goal of early detection and diagnosis of hemoglobinopathies.

Algorithms for Hemoglobinopathy Detection

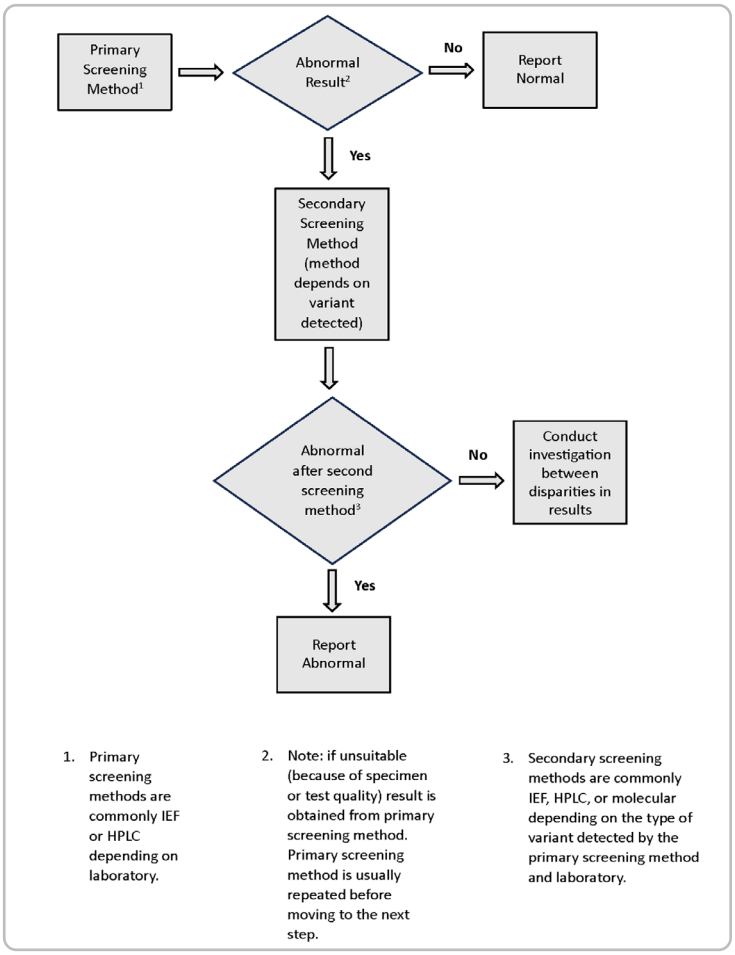
NBS programs may have varying approaches to screening and reporting, but the goal is the same: screen for those individuals who may be at risk for clinically significant hemoglobinopathies where early intervention leads to improved outcomes.

Basic Hemoglobinopathy Screening Process

The basic screening process entails use of a primary method to first screen all specimens, with most laboratories then reflexing to a secondary method depending on the type of abnormal hemoglobin or ambiguous result initially detected (**Figure 16**). Reporting follows based on the result obtained.

Methods differ in the ability to detect different hemoglobins, so understandably programs will also differ in what is typically reported due to method limitations. Additionally, programs may have different distribution of hemoglobins reported based on their states' priorities (see **Appendix B** for illustrations of nationwide variations in populations).

Figure 16. Basic Hemoglobinopathy Screening Process



Unknown Hemoglobin Variants

Invariably, NBS programs will identify unknown hemoglobin variants that may or may not be clinically significant. Most hemoglobin variants do not cause serious health problems, with the exception of a few. National consensus has yet to be established to guide NBS programs and clinicians in the follow-up of infants with unidentified hemoglobin variants. Final identification of unknown variants can be achieved through DNA analysis and consultation with a hemoglobin specialist for possible clinical implications.

Once a variant hemoglobin is identified through screening, it should be confirmed by a secondary or alternate method. The American College of Medical Genetics has created a set of ACT Sheets and Algorithms as a resource for healthcare providers.

Both IEF and HPLC carry the risk of misclassifying some hemoglobinopathies. For example, babies with S/beta-plus thalassemia may be mistakenly reported as having sickle cell trait (FAS). A small number of babies inheriting the sickle gene and deletional type hereditary persistence of fetal Hb (HPFH) with pancellular distribution of Hb F shows FS on these screens, consistent with sickle cell anemia (SS), but do not have features of SCD, like the vaso-occlusive crises or hemolytic anemia.[36]

Similarly, it can be difficult definitively classifying alpha or beta thalassemias without molecular confirmation.

Quality Assurance for Hemoglobinopathy Screening and Testing

NBS is an entire system, beginning at the hospital and continuing through to the diagnosis of the child. Each part of the NBS system is important and needs evaluation of any weaknesses. Regardless of the screening algorithm employed, a laboratory program must consider and institute quality assurance and quality control (QA/QC) activities.

QA is defined as, "a program for the systematic monitoring and evaluation of the various aspects of a project, service, or facility to ensure that standards of quality are being met" and is often split into three categories: pre-analytic, analytic and post-analytic.[37] Pre-analytic, analytic and post-analytic QA is crucial to the proper function of the laboratory system and reporting of quality and accurate screening results with clear indications of the possible genotypes that could be found upon confirmatory testing.

This section focuses on the analytic phase and briefly describes issues in the pre-analytical and post-analytical phases.

If more information about quality management is necessary, there are many resources including consensus documents from CLSI, World Health Organization's *Laboratory Quality Management System Handbook* and accreditation organizations such as ISO.

Pre-analytic Phase

For NBS, this phase covers all the steps taken prior to testing any NBS samples, including:

- The test request
- Specimen collection and quality
- Handling and transport to the testing facility within 24 hours of collection
- Specimen storage.

Performance evaluation indicators may include the percentage of unsatisfactory specimens (as determined by each program) received and the percentage of specimens that are delayed in transport.

Analytic Phase

This phase includes all factors related to laboratory testing. A laboratory's QA program should follow any applicable regulatory guidelines, but, at a minimum, should generally include the following components:

- A standard operating procedure detailing the testing method, how to interpret results and how to resolve problems that may arise
- Flow charts/responsibilities/actions to clarify the role of each person involved in the analysis
- QC materials to monitor the method over time
- Annual competency assessments and reviews of staff
- Proficiency testing (PT), an external quality assessment to monitor the laboratory's performance
- Routine instrument maintenance
- Documentation of any corrective actions taken.

Follow your quality management system plan. No QC materials in the DBS matrix are available for hemoglobinopathy screening.[38] The most useful QC and PT materials are those in the same matrix as the specimens being tested. The materials should include specimens classified as normal as well as those with hemoglobinopathies. Because of the high amount of fetal hemoglobin present in newborn DBS, PT for hemoglobinopathy screening requires umbilical cord blood to prepare DBS. Availability of umbilical cord blood with hemoglobinopathies is scarce and there are only two programs that provide PT materials: CDC's NBS Quality Assurance Program (NSQAP) and the United Kingdom National External Quality Assessment (UKNEQAS). If the PT program does not provide specimens for the hemoglobinopathies tested, laboratories may follow guidelines published in CLSI's *QM24-Ed3: Using Proficiency Testing and Alternative Assessment to Improve Medical Laboratory Quality*.

For SCD and other hemoglobinopathies, NSQAP sends panels of five blind-coded DBS PT specimens to participating laboratories three times per year. Participants are asked to test the panel and for each specimen, report the method(s) used, the presumptive phenotype, the presumptive clinical assessment and any other clinical classifications that are consistent with their program operations. An individualized evaluation is provided back to each participant. NSQAP participating laboratories can download summary reports from the NSQAP Participant Portal.

If a participant has an error in a PT survey, the laboratory should investigate the error, determine root cause and implement corrective measures to reduce the risk of the error happening again. Due to the limited number of specimens available, there is currently a waiting list to join this program.

UKNEQAS provides a PT program for sickle cell and other abnormal hemoglobinopathies in DBS. It provides three specimens each month. For further information, please visit the UKNEQAS website.

Post-analytic Phase

The post-analytical phase involves all aspects following completion of the specimen analysis:

- The requirements of the NBS laboratory results comments
- Report results to submitters and provide access to results to provider of record
- The transmittal of the screening abnormal laboratory results to the follow-up team to initiate the confirmatory process
- The determination of what actions were taken regarding positive screen results
- Continual monitoring of confirmatory feedback on presumptive positive screens.

Communicating positive screen results to a doctor and/or parents and tracking outcomes of diagnostic testing are of primary concern. Follow-up processes for NBS primarily aim to quickly locate an infant with SCD for diagnosis and treatment. Although protocols vary between programs, the subsequent information focuses specifically on SCD follow-up versus non-disease reporting and trait reporting. Additionally, varying algorithms from state programs have been included as examples (See **Appendix D** and **Appendix E**).

Follow-up

The goal of follow-up is to ensure that all who receive a valid screening test and screen positive results, receive a definitive diagnosis in the most expedient manner possible and appropriate clinical management, if confirmed.[39] To ensure this is achieved, the following actions must be performed:

- Access to the newborn within days of birth or to non-newborns, when possible
- Aid in communication of screening test results to the clinician
- Notification and dissemination of screening results to appropriate personnel required to facilitate achievement of the primary goal
- Referral to primary healthcare provider and/or specialist
- Collection of adequate blood and prompt submission to the designated confirmatory laboratory
- Diagnostic testing to confirm screening test results
- Establishment of comprehensive care in a medical home
- Initiation of education as well as penicillin prophylaxis for SCD or therapies for other hemoglobinopathies
- Ensure information, services and supports for families are accessible, timely and effective
- Advance understanding of outcomes for children with hemoglobinopathies and other NBS conditions through the collection, integration and evaluation of key data
- Evaluate and build NBS system capacity and promote equitable access to interventions.

For sickle cell trait, some programs refer to a specialist or genetic counselor for confirmatory testing.

While follow-up of those with presumptive SCD has a clear purpose, the purpose of follow-up of those with other hemoglobinopathies varies and should be discussed with a hematologist. Successful implementation of follow-up involves the following:

- Productive relationships and communications between the screening laboratory and follow-up staff
- Selection and training of staff reporting results to the family, providers and specialists
- Processes or algorithms for follow-up to include confirmation testing and education.

Relationships Between Laboratory and Follow-up Staff

In some NBS programs the screening laboratory reports only to staff of the state NBS program to which all results are transmitted. In others, the laboratory also reports to the hospital of birth and to the listed primary healthcare provider for the baby. Since NBS for SCD is uniformly state-mandated, the state is responsible for implementation and monitoring of the screening program, or its contracted follow-up agency. Either entity should be the recipient and distributor of screening results. Increasingly, follow-up staff and healthcare providers have electronic access to the laboratory results held by the state.

By whatever method of communication between laboratory and follow-up staff, two issues are important: first, no single screening test can establish with certainty the phenotype of all the common types of SCD and related conditions, and second, staff at the laboratory and follow-up agency must be fully familiar with the nomenclature of Hb phenotypes and result comments reported by the lab, their clinical significance, and guidance to the clinician.

Selection and Training of Staff Reporting the Screening and Confirmatory Results to the Family

Reporting results for potentially affected individuals and reporting about those with non-disease conditions should be fundamentally different. Training should equip staff with basic knowledge about SCD and related thalassemia conditions, their variants and their inheritance patterns and the difference between benign carrier states and clinically significant diseases.

Staff who are reporting results for affected individuals should be familiar with the clinical course of SCD in infants and young children, be able to answer basic questions about SCD or the suspected related disease and be ready to provide reassurance and support for the family. If staff are not familiar with children with SCD, their training should include an internship at a Sickle Cell Center where they can observe children with SCD in both outpatient and inpatient settings. This will enhance and make more realistic the knowledge acquired through instruction or reading and video learning materials. For additional questions, please navigate to the Hemoglobinopathies Project webpage and email the Hemoglobinopathies Workgroup.

The purpose and value of counseling related to a baby with sickle cell trait or other clinically benign hemoglobin conditions or to a baby with no abnormal Hb on NBS is not always clear. Any family whose newborn is tested for any disorder deserves to receive results and have their implications explained. This could be done through staff at a primary health care facility, or another agency contracted by the state. However, several programs imply, by design, that NBS opens a window for genetic education and counseling of the parents for future reproductive planning. Follow-up staff reporting NBS results must have some knowledge of the genetics of hemoglobin disorders.

Process or Algorithms for Follow-up

Affected Individuals

Notification of a family about a (presumptive) serious health problem in an otherwise healthy appearing newborn is not a trivial task. Ideally, notification should be done in person or by live phone call. The initial conversation should be expected to raise anxiety, and cause disappointment and sadness at a time of joy; it may also stir anger or denial. The messenger should be someone familiar with the clinical course of SCD in infants, young children and adults, and be able to answer basic questions about SCD and ready to provide reassurance and support for the family. Verbal notification should be followed with educational materials and resources for families to obtain more information.

The processes and algorithms for notification and follow-up often place the primary care service as the medical home, presuming that staff can interpret screening results and take appropriate actions when a baby with a positive screen is reported. Reporting individuals with abnormal screening results to a dedicated follow-up agency and/or sickle cell treatment center will increase the timeliness of appropriate care.

In the best circumstances, each child with presumptive SCD should be assigned to one case manager who handles the baby from screening test results through establishment at the medical home and sickle cell treatment center, where available.

Non-affected Individuals

All parents should be informed about the results of NBS. Informing parents about results of NBS that carry no clinical significance should be handled with calmness and reassurance, and an explanation that it is a screening test. Algorithms for reporting and following up babies with HbFA on screening should not classify them as “Normal” or “Within Normal Limits;” they should be reported as “No Abnormal Hb Found.”

“Trait Counseling” of parents of heterozygous babies has become an integral part of NBS programs for hemoglobin disorders. However, an FA baby may be born to parents who BOTH have sickle cell trait or, one may have sickle cell trait while the other is a carrier of another abnormal hemoglobin or beta thalassemia. Such parents can have a subsequent baby who has homozygous Hb SS or another type of SCD. At risk parents should become aware of their hemoglobin genotypes.

Adults Who Screen Positive for a Hemoglobinopathy Trait

It is important to screen at-risk individuals for hemoglobinopathies and who may not have been screened at birth.

Dissemination of information about the relevance of adult screening and participation in community outreach activities, such as community screenings and health fairs are very important for individuals who have not been previously tested.

If an adult individual has a positive diagnosis for a hemoglobinopathy trait, proper follow-up should ensure the understanding of the trait condition, with full explanation of the results, education about the trait (including any possible complications or rare clinical manifestations), counseling and the offering of hemoglobinopathy testing for the partner, if the couple is contemplating having children. Counseling should include the probability of the couple having a child with a hemoglobinopathy in each pregnancy.

Appendices

Appendix A: Examples of Molecular Methods for Hb Variant Detection

There are multiple techniques that can be used for defining variants molecularly, and the technology is constantly evolving. The most common approaches are real-time PCR-based allelic discrimination and sanger sequencing. Featured below are images of results seen for different hemoglobin patterns using allelic discrimination (**Figures A1, A2 and A3**). Additionally, images of sanger sequencing (**Figure A4**) are included to demonstrate how variants can be identified by analyzing the sequence data (**Figure A5**).

Figure A1. **Allelic discrimination assay showing amplification of wild-type beta globin (solid line) and no amplification of the sickle cell variant (hatched line)**

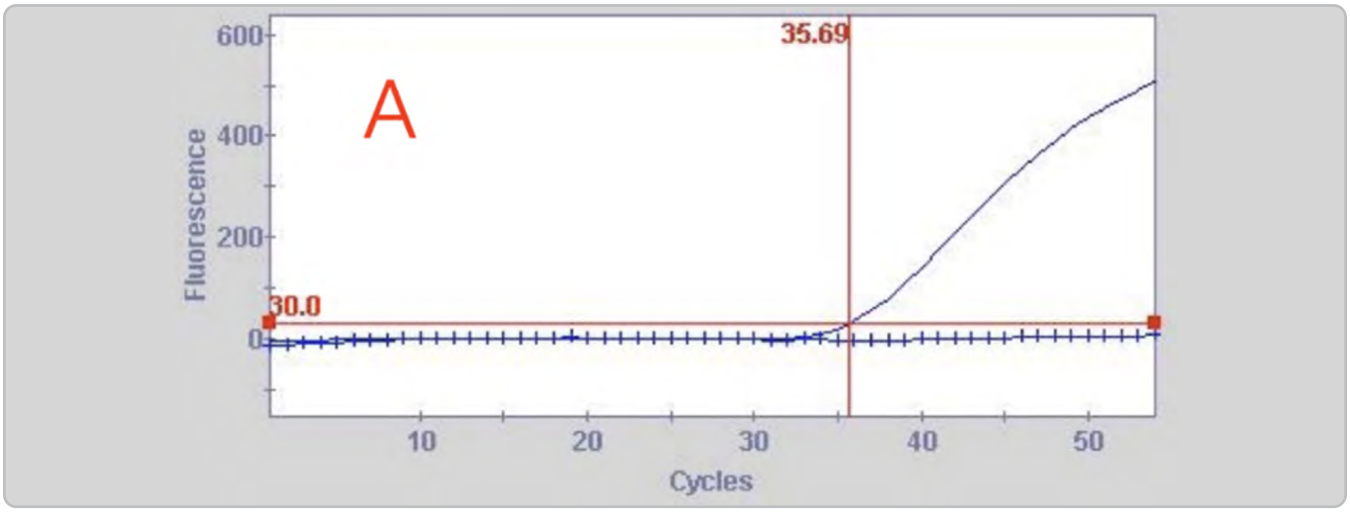


Figure A2. **Allelic discrimination assay showing amplification of both wild-type beta globin (solid line) and the sickle variant (hatched line) consistent with sickle cell trait**

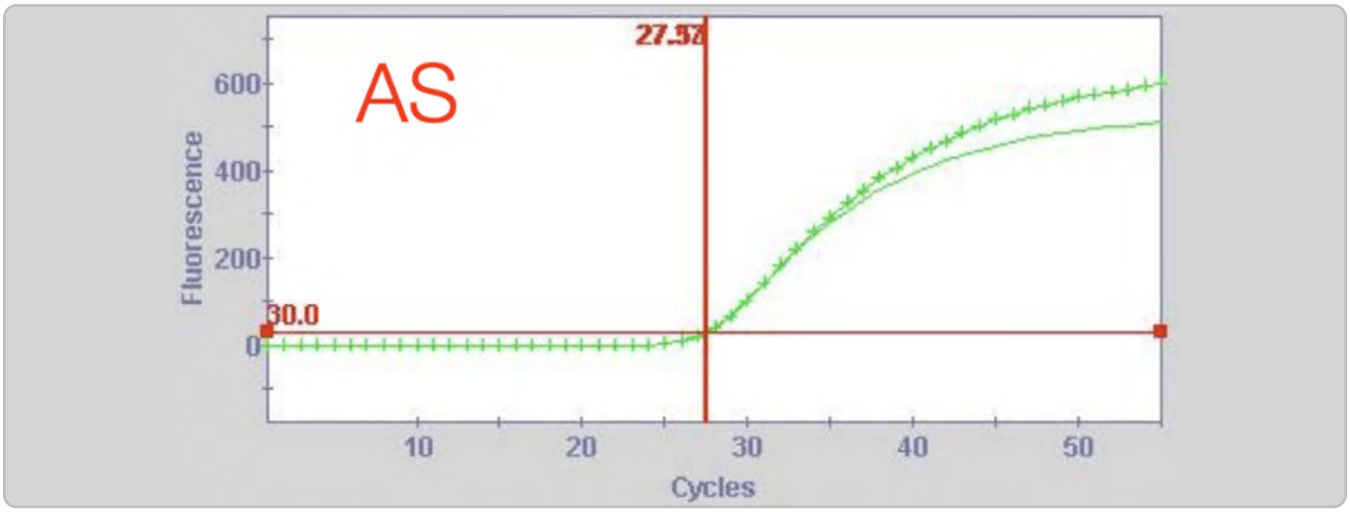


Figure A3. Allelic discrimination assay showing no amplification of wild-type beta globin (solid line) but amplification of the sickle variant (hatched line)

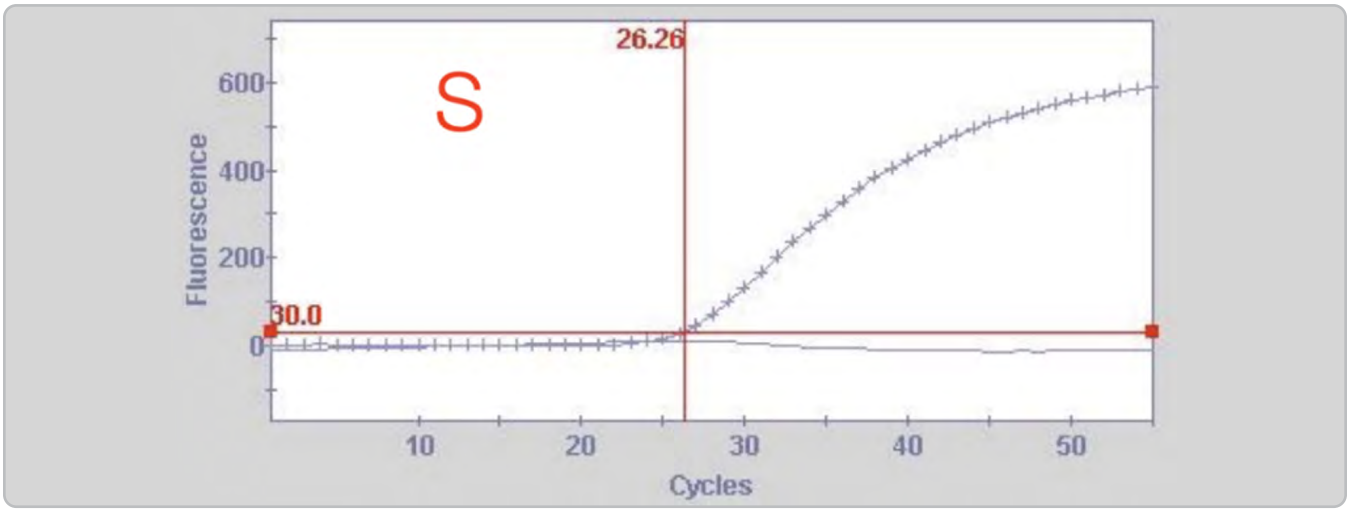
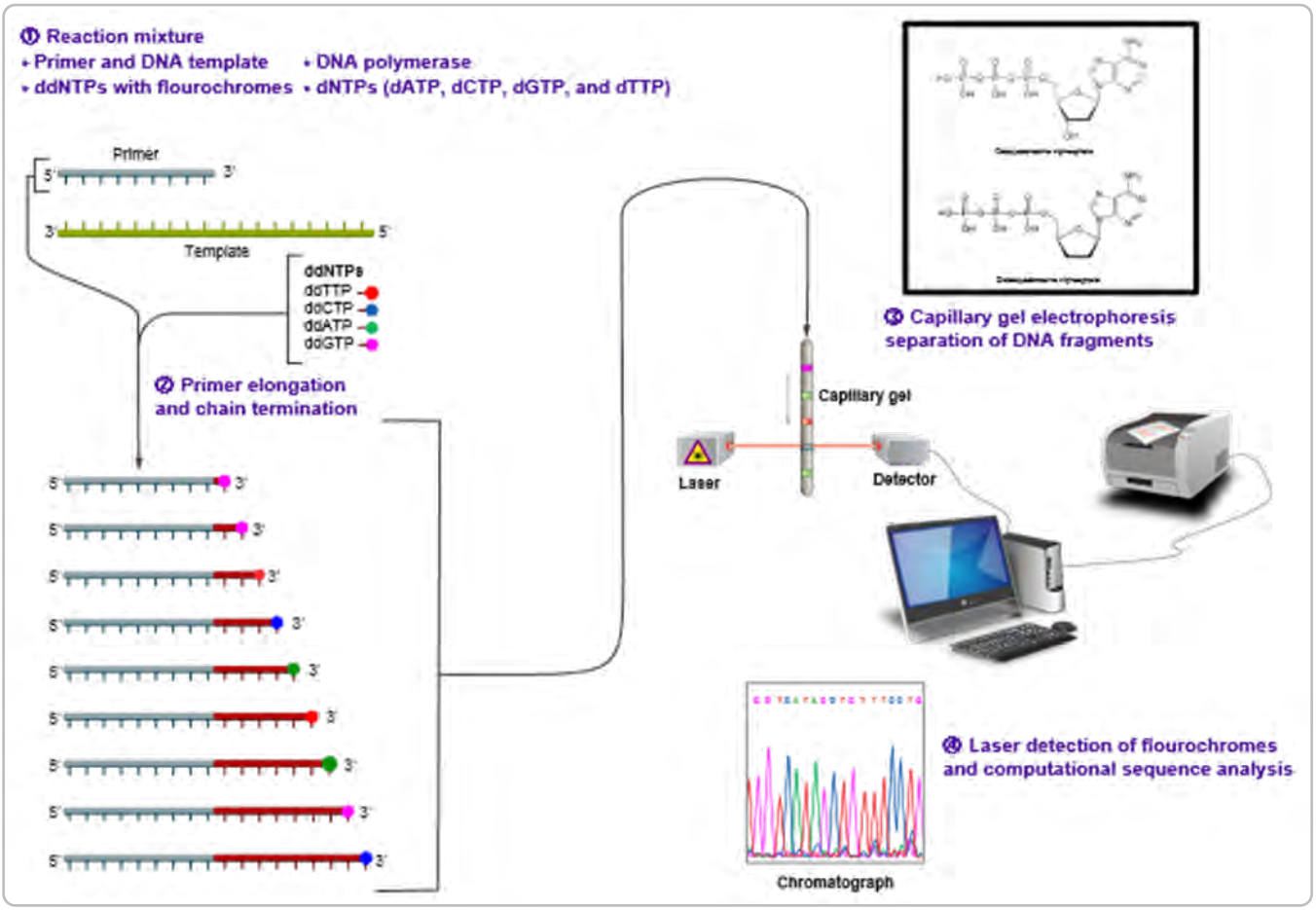
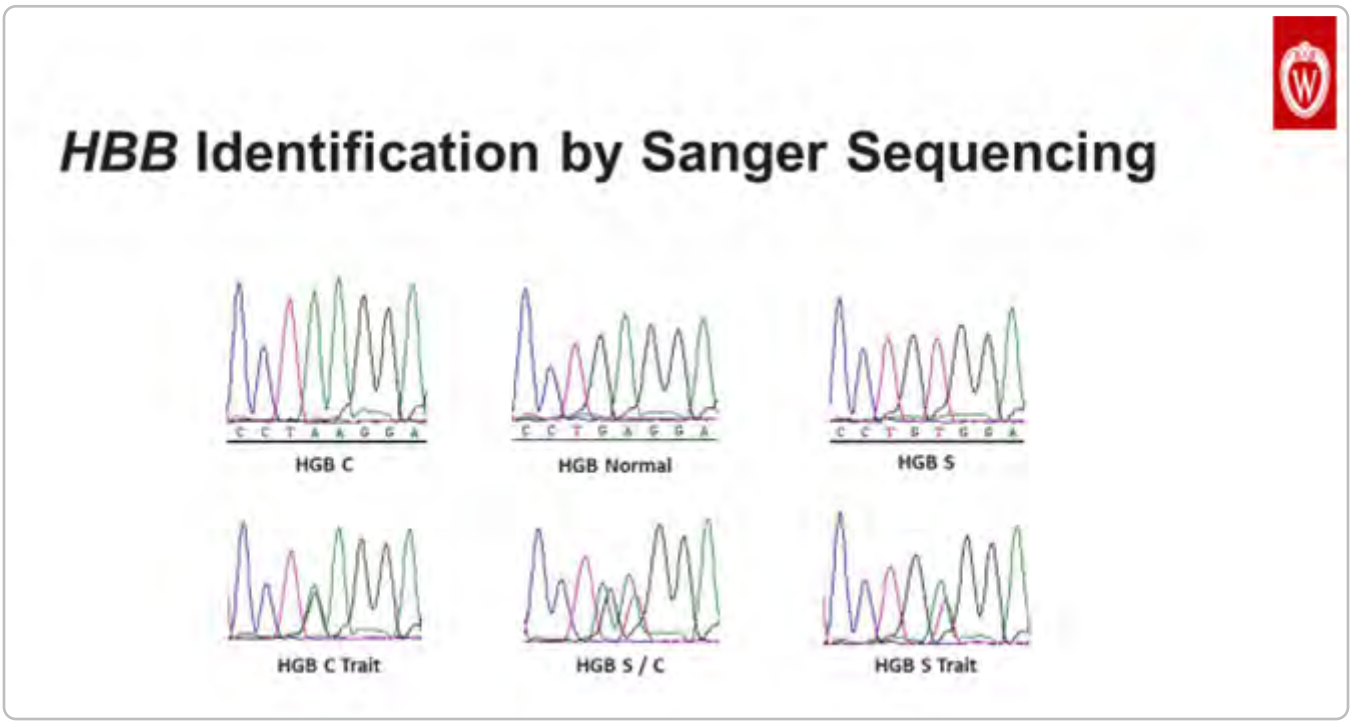


Figure A4. Depiction of Sanger sequencing* technology



* Source: Estevezj (Own work) [CC BY-SA 3.0 (http://creativecommons.org/licenses/by-sa/3.0)], via Wikimedia Commons

Figure A5. Chromatograms depicting six different hemoglobin patterns using Sanger Sequencing for the HBB gene; each chromatogram displays peaks corresponding to nucleotide sequences, with the bases labeled as C, T, G, and A at the bottom of each graph*



* Source: Mei Baker, MD, Wisconsin State Laboratory of Hygiene & Department of Pediatrics, University of Wisconsin, Madison, WI, US

Appendix B: Top Hemoglobinopathy Traits Identified in 2018–2022

Each chart (**Figures B1–B7**) below shows the frequency of the most common hemoglobinopathy traits reported from seven different NBS programs from 2018–2022. These charts highlight both commonalities and differences in the incidence of specific hemoglobin variants. Additionally, each program uses different mnemonics for the hemoglobin variants.

Also included in parentheses are links to the state coding systems—the result mnemonics and corresponding interpretations of the hemoglobinopathy phenotype—for four of the seven states. Notably there are some similarities and differences. These examples should not be seen as guidelines but rather should highlight the variability of mnemonics and the public health benefit of harmonizing. The state number in the chart corresponds to the state number coding system.

Figure B1. State 1 Top HgB Traits, 2018–2022 (State Coding System)

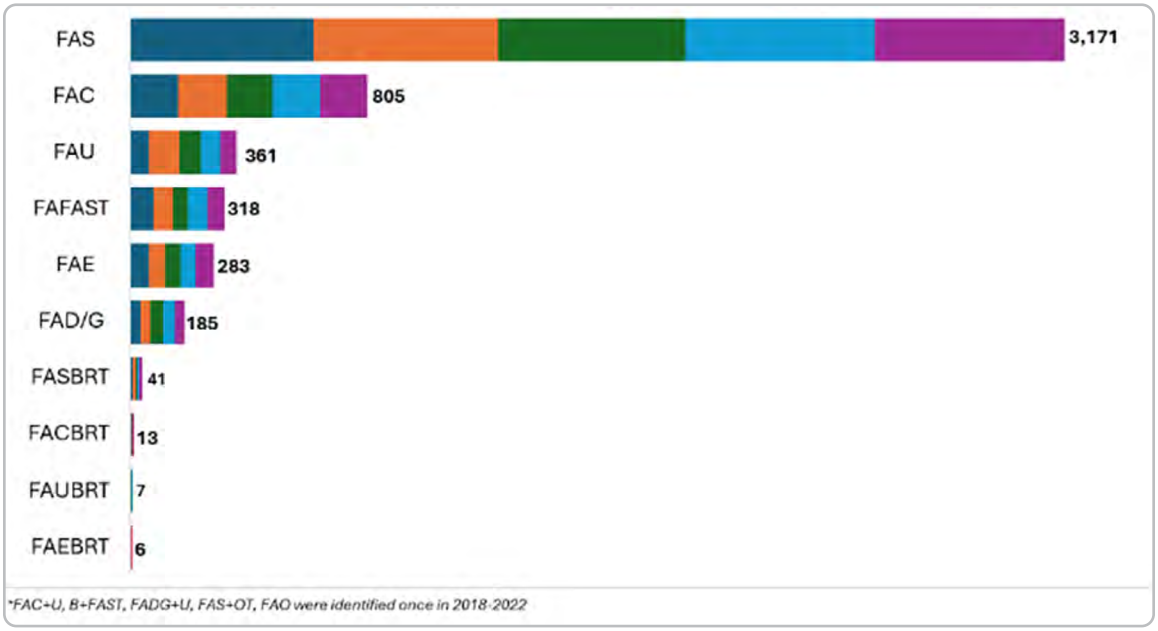
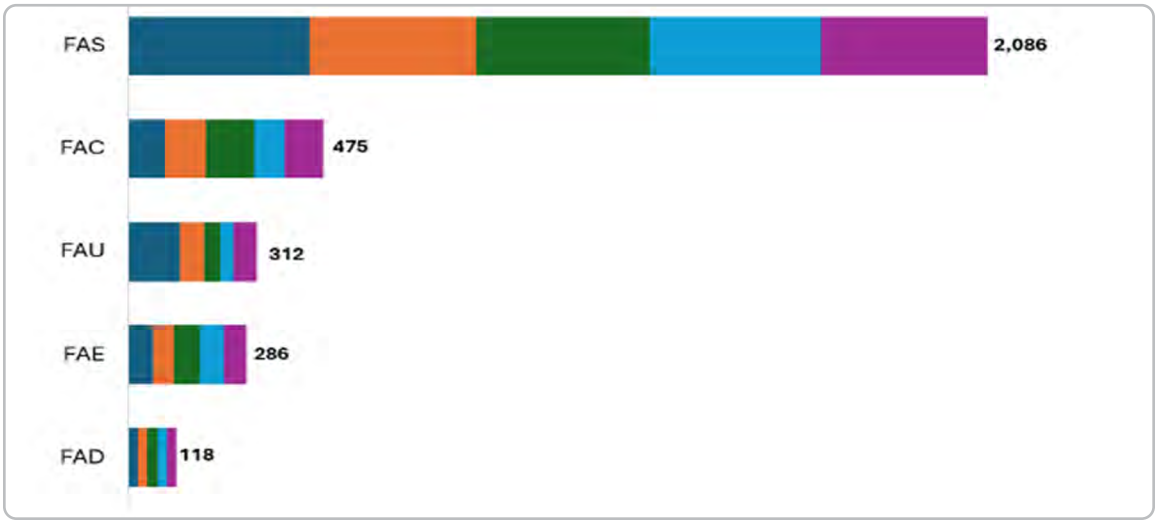


Figure B2. State 2 Top HgB Traits, 2018–2022



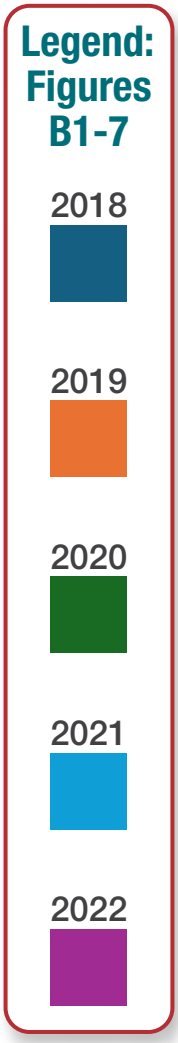
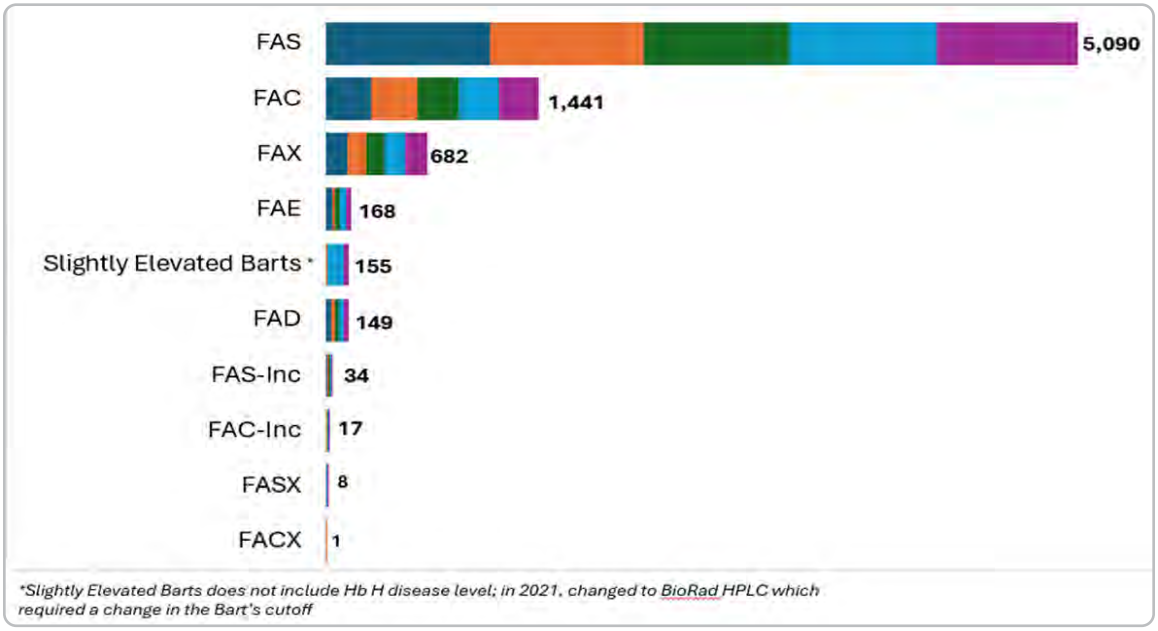


Figure B3. State 3 Top HgB Traits, 2018–2022 (State Coding System)

Figure B4. State 4 Top HgB Traits, 2018–2022 (State Coding System)



Legend: Figures B1-7

2018

2019

2020

2021

2022

Figure B5. State 5 Top HgB Traits, 2018–2022 (State Coding System)

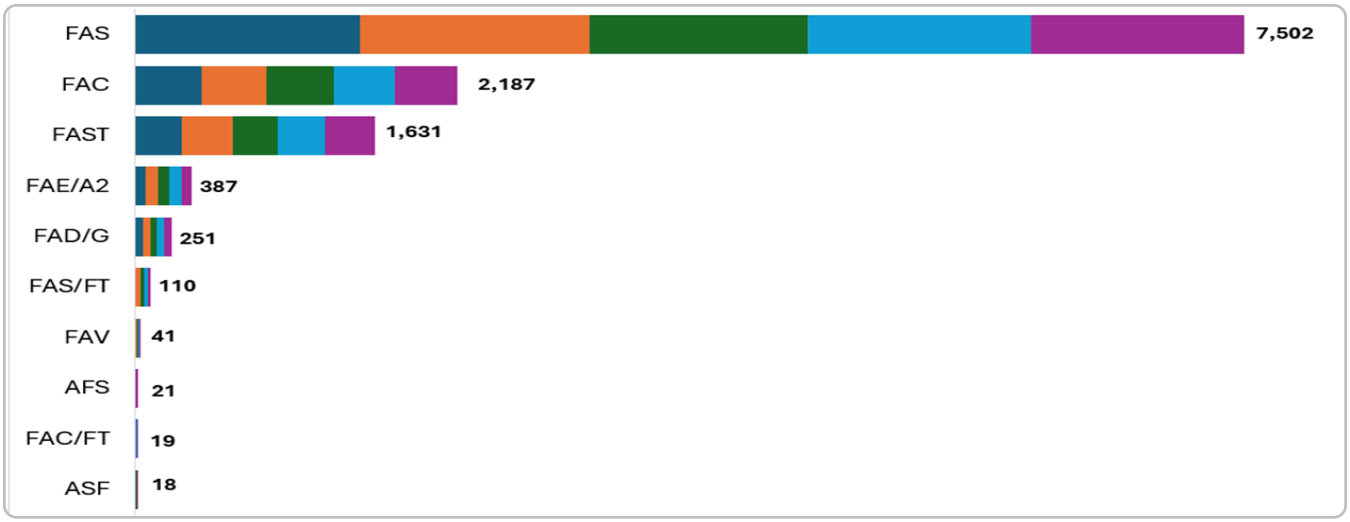


Figure B6. State 5 Top HgB Traits, 2020–2022

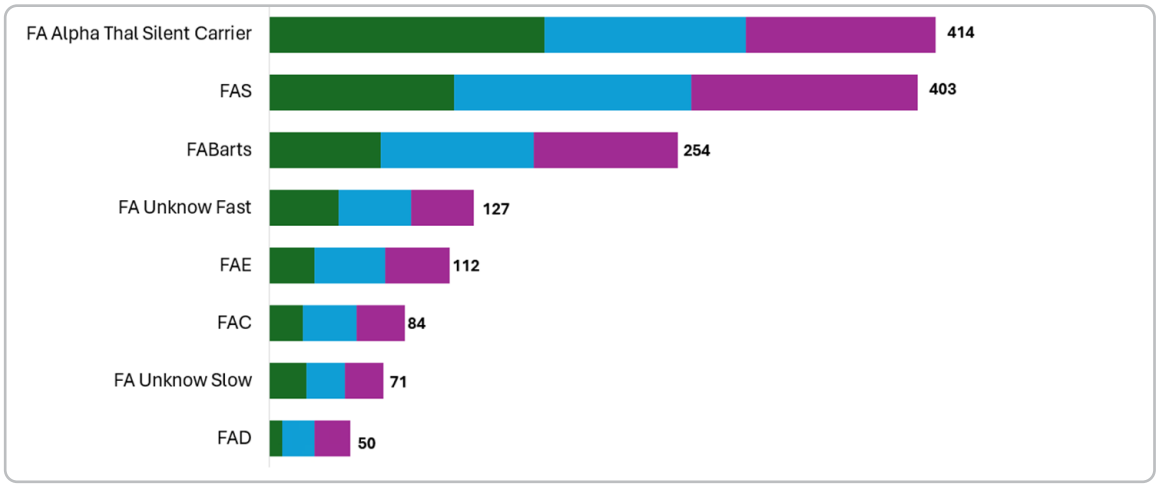
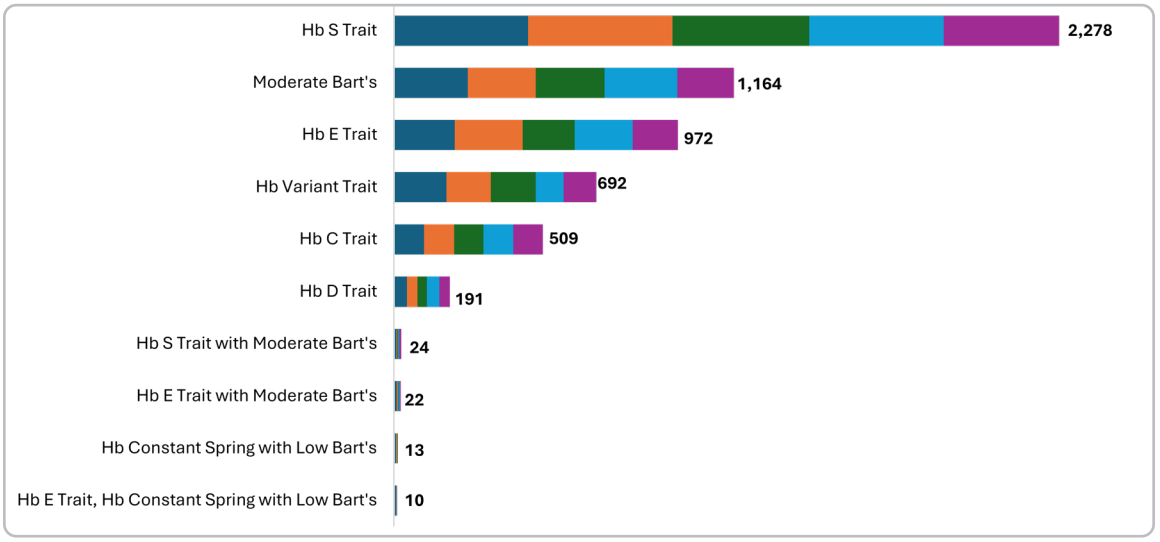


Figure B7. State 5 Top HgB Traits, 2018–2022



Legend: Figures B1-7

2018

2019

2020

2021

2022

Appendix C: Hemoglobin Patterns Reflexed to Molecular Screening

Below is a table used in one NBS laboratory listing the hemoglobin results that indicate reflexing to molecular screening as part of the NBS lab's algorithm.

✓ (T): sent for DNA testing only if demographic information shows the baby has been transfused.

Hemoglobinopathy Result	Forward to PCR (DNA)
F,A	-
F,A,S	✓ (T)
F,A,C	✓ (T)
F,A,D	✓ (T)
F,A,Other	-
F,S	✓
F,C	✓
F,S,C	✓
F,A,E	✓ (T)
F,E	✓
A,F	-
A,S,F	✓ (T)
A,C,F	✓ (T)
S,C,F	✓
S,F	✓
C,F	✓
E,F	✓
A,A	-
A,S	✓ (T)
A,C	✓ (T)
A,E	✓ (T)
A,D	✓ (T)
S,S	✓
S,C	✓

Hemoglobinopathy Result	Forward to PCR (DNA)
C,C	✓
E,E	✓
F,D	✓
F Only Detected	✓
F,S (A Questionable)	✓
F,C (A Questionable)	✓
F,E (A Questionable)	✓
F,A,S,Other	✓ (T)
A,E,F	✓ (T)
A,D,F	✓ (T)
A,Other	-
A,F,Other	-
A,F,S	✓
A,F,C	✓
F,Other	✓
F,A,Barts	-
F,A,S,Barts	✓ (T)
F,A,C,Barts	✓ (T)
F,A,E,Barts	✓ (T)
F,A,D,Barts	✓ (T)
F,A,O-Arab	✓ (T)
F,A,elevated Barts	-
F,S,Barts	✓
F,S,A	✓

Hemoglobinopathy Result	Forward to PCR (DNA)
F,C,A	✓
F,S,C,Barts	✓
F,A,Other,Barts	-
A,F,Other,Barts	-
A,F,S,Barts	✓
A,F,D	✓
A,G	-
F,G	-
A,G,F	-
F,A,G,Barts	-
A,F,G	-
F,A,C,Other	✓ (T)
S,A	✓
C,A	✓
F,A,G	-

Appendix D: State Hemoglobinopathy Screening Algorithms

Presented below are two examples of labs' flowchart outlining the steps and criteria for reflex testing and result determination. Both examples use IEF, HPLC and molecular methods, however the sequence differs. The process in **Figure D1** summarizes which phenotypes are reflexed after IEF to HPLC and then which are reflexed to molecular screening. In **Figure D2**, an abnormal IEF result can reflex to short gradient, extended-gradient and molecular screening. The type of testing for each hemoglobin phenotype is indicated in **Figure D2**.

These examples should not be seen as guidelines and are intended to provide examples of current practices in hemoglobinopathy screening using tiered methods.

Figure D1. **Flowchart outlining the criteria for reflex testing used by NBS laboratory X**

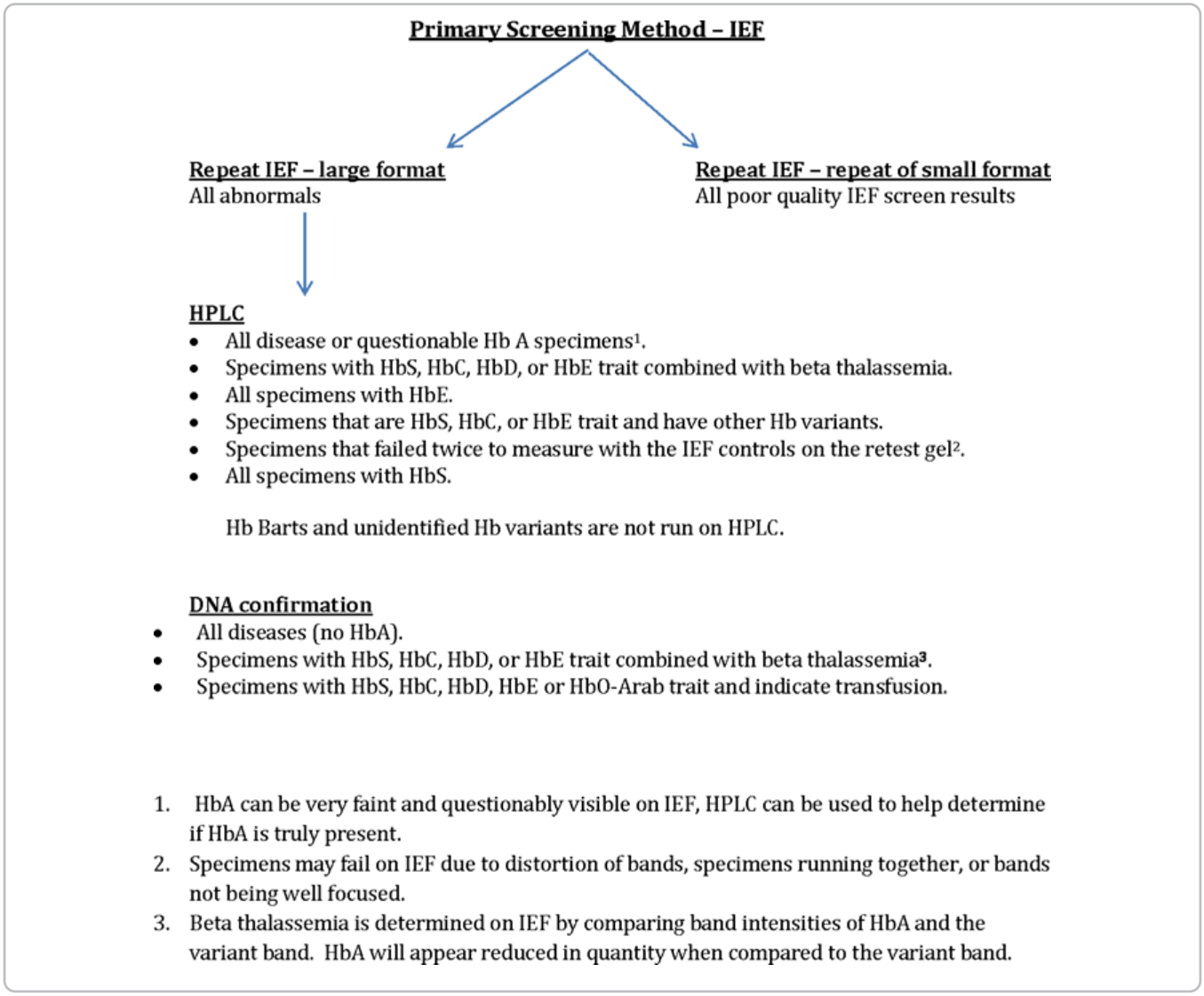
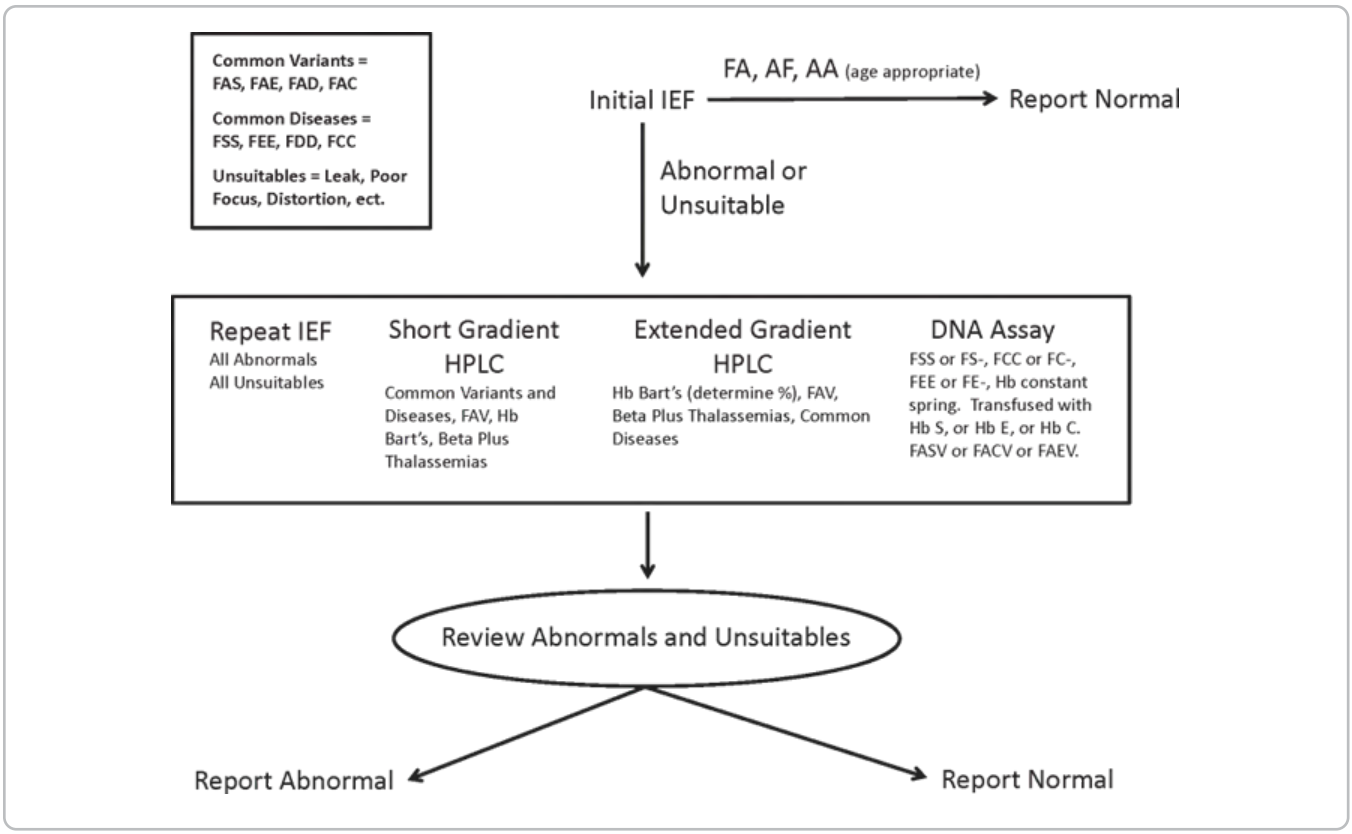
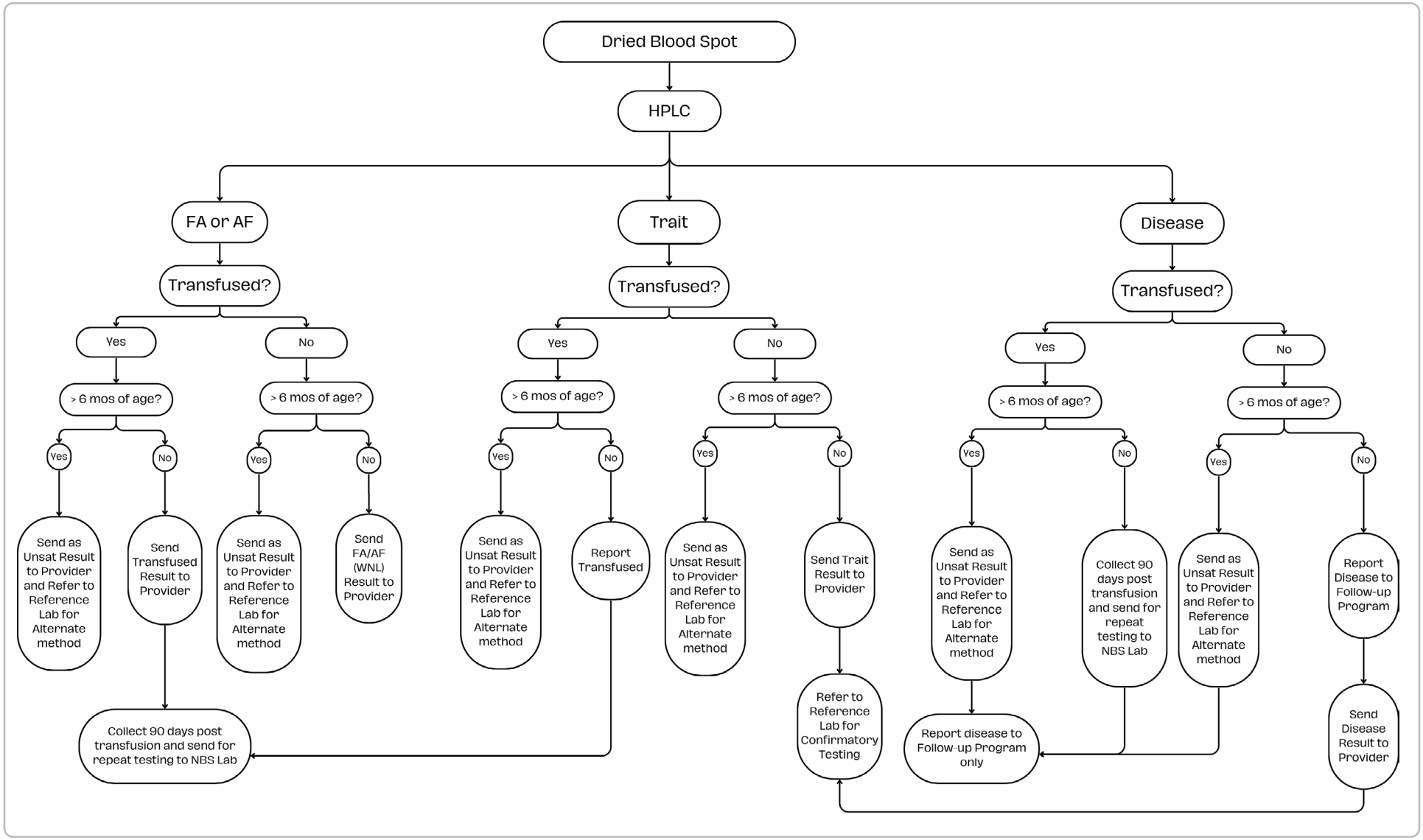


Figure D2. Flowchart outlining the criteria for reflex testing used by NBS laboratory Y



Appendix E: State Hemoglobinopathy Reporting Algorithm

Below is an example of one state's process for reporting results to providers and follow-up. The flowchart walks users through the different scenarios with guidance on the next steps.



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