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Specimen repository for evaluating new point-of-care tests for G6PD deficiency

A resource for accelerating diagnostic product development

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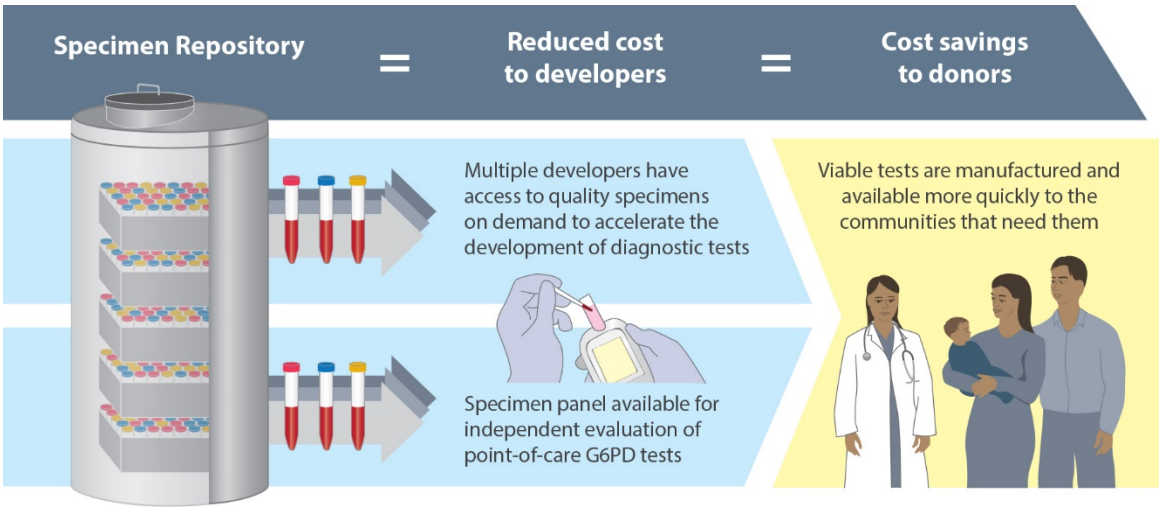
Executive summary

People with deficiencies in the metabolic enzyme glucose-6-phosphate dehydrogenase (G6PD) are at high risk for severe anemia and its consequences if treated with certain anti-malaria drugs, the 8-aminoquinolines. These medications are used to kill the *Plasmodium vivax* (*P. vivax*) parasite, which causes about half of malaria cases outside sub-Saharan Africa. More than 400 million people worldwide have G6PD deficiencies, which are especially common in malaria-endemic regions. Best clinical practice requires testing for G6PD deficiency before administering the drugs, but tests that meet technical requirements and are appropriate for use in low-resource settings are not currently available. PATH aims to accelerate the development and introduction of a point-of-care (POC) G6PD diagnostic test to support the safe use of 8-aminoquinoline-based drugs in areas with *P. vivax* malaria.

Test developers will need to evaluate their technologies throughout the product development process with blood samples expressing different G6PD levels that have been characterized by gold standard G6PD assays. However, collecting, processing, and maintaining a supply of samples would be costly and time-consuming for individual companies. Obtaining specimens with a range of G6PD activities, from severely deficient to normal, is challenging because of the low prevalence of mutations in non-malaria-endemic regions, where most test development occurs.

To address this need, PATH has established a repository of cryopreserved blood samples highly characterized for G6PD activity. The cryopreservation technique keeps red blood cells from rupturing and prevents loss of enzyme activity, so that cells can be analyzed weeks to months after blood collection. The specimens, recruited on a continuous basis, will be used at PATH to evaluate performance of the technologies as they progress through product development stages. Panels of specimens also are available to all product developers, ensuring global access to the resource and facilitating an accelerated G6PD diagnostic product pipeline.



Introduction

Malaria mortality rates have fallen by more than 45 percent globally since 2000[1], but progress toward elimination has slowed. One of the barriers to elimination is the difficulty of destroying all life stages of the *P. vivax* parasite, which is responsible for 50 percent of malaria cases outside of sub-Saharan Africa. *P. vivax* forms hypnozoites—dormant liver stages—that can remain undetected for months to years and emerge periodically to cause relapses, rendering patients ill and infectious again. Getting rid of this reservoir of *P. vivax* hypnozoites is essential for malaria elimination.

One class of drugs—the 8-aminoquinolines—is effective in destroying hypnozoites, a process termed radical cure, but can cause hemolysis (rupture of red blood cells) in patients with a deficiency of the metabolic enzyme G6PD. This deficiency occurs in 400 million people worldwide and is especially common in malaria-endemic regions (Figure 1).

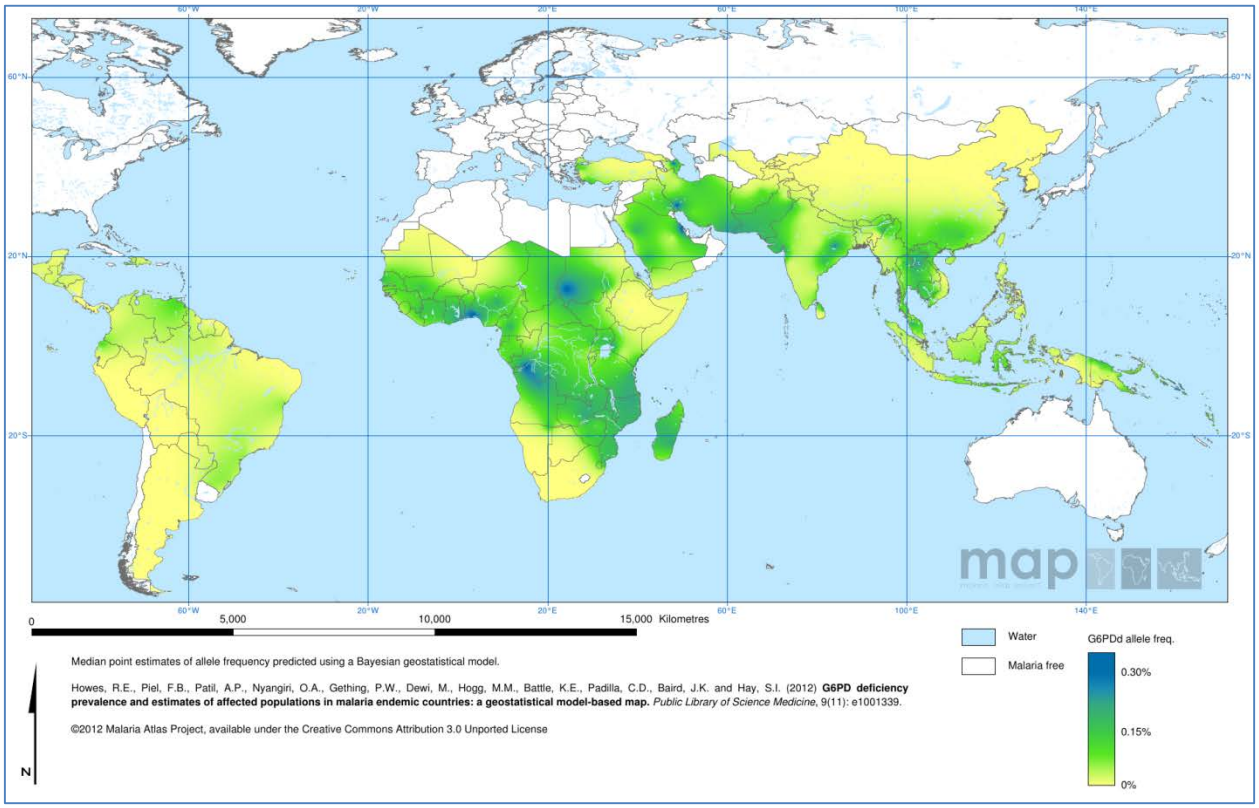


Figure 1. Predicted frequency for G6PD deficiency within malaria-endemic countries in 2010.[2]

Estimates were made based on data from community surveys that were used to give gene frequency estimates and adapted to reflect the X-linked inheritance of the G6PD gene.
http://www.map.ox.ac.uk/browse-resources/g6pd/G6PD_freq/world/

While all people exposed to 8-aminoquinolines experience some drop in hemoglobin concentration, those with G6PD deficiency are more likely to experience hemolytic anemia with the sequelae of kidney damage, blood-flow compromise and, possibly, death.[3,4] Primaquine, a drug in this class, has been in use since the 1950s without a standard requirement for G6PD testing prior to use because of its adoption in an earlier era with a different drug regulatory climate. A long-acting 8-aminoquinoline, tafenoquine, is in

development, with the goal of providing radical cure with one dose instead of the current 14-day dosing regimen for cure with primaquine. Best clinical practice requires testing for G6PD deficiency before primaquine or tafenoquine are administered, but tests that meet technical requirements and are appropriate for use in low-resource settings are not currently available.[5]

With funding from the United Kingdom's Department for International Development and the Bill & Melinda Gates Foundation, PATH is working with the private sector to support the development of POC tests for G6PD deficiency. Test developers will need to evaluate their technology on an ongoing basis by assessing performance with blood samples that have been characterized by gold-standard assays; however, collecting, processing, and maintaining such a supply would be costly and time-consuming for individual companies. PATH has established a process and a repository to fulfill this need.

Specimen repository

Rationale for a G6PD-characterized blood specimen repository

A critical need for development of any diagnostic product is access to characterized clinical specimens representing the dynamic range and clinical presentations the target product must be able to differentiate. For G6PD test development, obtaining specimens with a range of G6PD activities, from severely deficient to normal—including specimens from heterozygous females[a]—is challenging because of the low prevalence of mutations in non-malaria-endemic regions. Blood-collection centers in the United States typically find only a few deficient samples in a hundred donations. Having each test developer source blood specimens is inefficient, because the quantity of blood collected in a single blood draw can support many evaluation studies—the cost of providing specimens and their characterization data to additional parties is low.

To support work on new POC G6PD tests, PATH has established a unique specimen repository of cryopreserved blood samples highly characterized for G6PD activity; that is, several assays are performed to determine enzyme activity accurately, as discussed below. Making these specimens available to test developers will support the development and subsequent evaluation of new diagnostic devices. PATH will monitor product development investments by evaluating prototypes from different developers using the same specimen panels to compare test sensitivity, accuracy, and precision. Creating this repository required that PATH establish standard methods for cryopreservation to stabilize G6PD activity in intact red blood cells. The next section discusses this process and the analyses performed to characterize specimens for evaluation of G6PD tests.

Analysis and cryopreservation of specimens

Since blood specimens deteriorate within one to two weeks even when refrigerated, they must be preserved if a functional repository is to be maintained. To this end, PATH developed and published a technique for stabilization and cryopreservation of intact red blood cells.[6] Our work demonstrated good correlation between G6PD activities in fresh and cryopreserved specimens. Cytochemical staining showed that intracellular G6PD activity for individual red blood cells also was maintained, and the

[a] The G6PD gene is on the X-chromosome, so females have two copies, but males have only one (and one Y-chromosome). Females thus have two populations of red blood cells, which can have different enzyme activity levels.

mosaic composition of red blood cells in heterozygous women was preserved for at least six months (Figure 2). With these cryopreserved specimens, G6PD activity in intact cells can be analyzed weeks to months after blood collection, whenever test developers need to evaluate prototypes.

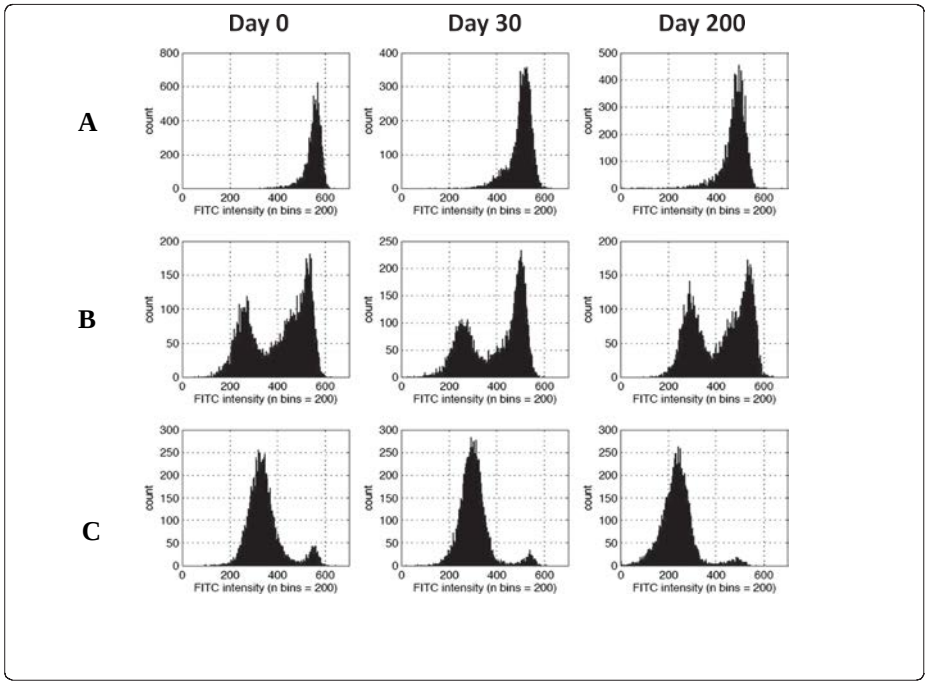


Figure 2. Distribution of G6PD activity in red blood cell populations of fresh and cryopreserved specimens.

G6PD activity was measured indirectly by a cytochemical staining assay. Intracellular activity distributions are shown for (A) normal male, (B) heterozygous female, and (C) deficient male specimens. G6PD activity profiles are shown for Day 0 prior to cryopreservation and two separate dates at which the same specimen was thawed after cryopreservation.

Specimen repository workflow

Specimens are sourced from blood-collection centers in the United States, with the aim of procuring donors from a wide range of ethnic origins to ensure diverse representation of G6PD mutations. Samples—each about 20 milliliters (mL) in volume—are collected in an anticoagulant to protect cells and preserve enzymes and are shipped overnight under refrigeration to the PATH laboratory. The chain of custody of samples is maintained by a laboratory database management system (Freezerworks Unlimited, Mountlake Terrace, WA, USA). After a stabilizing solution of sugars, salt, and adenine is added to specimens, their G6PD activity is characterized on a small portion of the sample, using three different tests. The Trinity Biotech quantitative assay (Trinity Biotech Plc, Bray, Ireland) and the Trinity Biotech qualitative fluorescent spot test both use lysed red blood cells—the membranes of the cells are ruptured and contents released—and measure average G6PD activity in all the cells. The third test, a cytochemical staining test, is used to observe G6PD activity in intact cells and allows detection of females with heterozygous G6PD mutations. This assay uses flow cytometry to reveal whether there are two red blood cell populations—with different enzyme activity levels—in an individual female.

After blood specimens are characterized with the three assays noted above, portions of each are processed and preserved as follows:

- Specimen type 1: Intact red blood cells are cryopreserved in liquid nitrogen according to established methods.[6] Maintaining cell integrity has two critical implications: 1) the specimens can be used to fully evaluate a G6PD test, including use of assays that require cell lysis; 2) the specimens have a long (> 5 days) shelf life at 4°C, so they do not need to be used immediately upon thawing. This specimen type is of most value to test developers for in-house assessment and for rapid evaluation at PATH of POC tests received from developers.
- Specimen type 2: Whole blood is frozen in small volumes, or aliquots, and stored in liquid nitrogen without a cryopreservation technique. Aliquots of this type will lyse upon thawing and can be used for qualitative and quantitative tests to determine G6PD activity. These specimens must be used immediately upon thawing.
- Specimen type 3: Peripheral blood mononuclear cells (white blood cells) are preserved in liquid nitrogen for DNA sequencing. This analysis will show whether the donor has a mutation in the G6PD gene.[b]

For specimen panels that will be sent out to developers, the process begins with the PATH lab thawing aliquots of the specimens and performing the same three critical G6PD assays used for initial characterization, to ensure that G6PD activity was not compromised during cryopreservation. After this quality-control step, the thawed specimens are shipped to the developer under refrigeration. These highly characterized samples will enable manufacturers to validate their tests by comparing results with data from the PATH laboratory on the same specimens. Alternately, PATH will host the product developers at its facilities to provide them access to the specimens under confidentiality. The specimen repository workflow is illustrated in Figure 3.

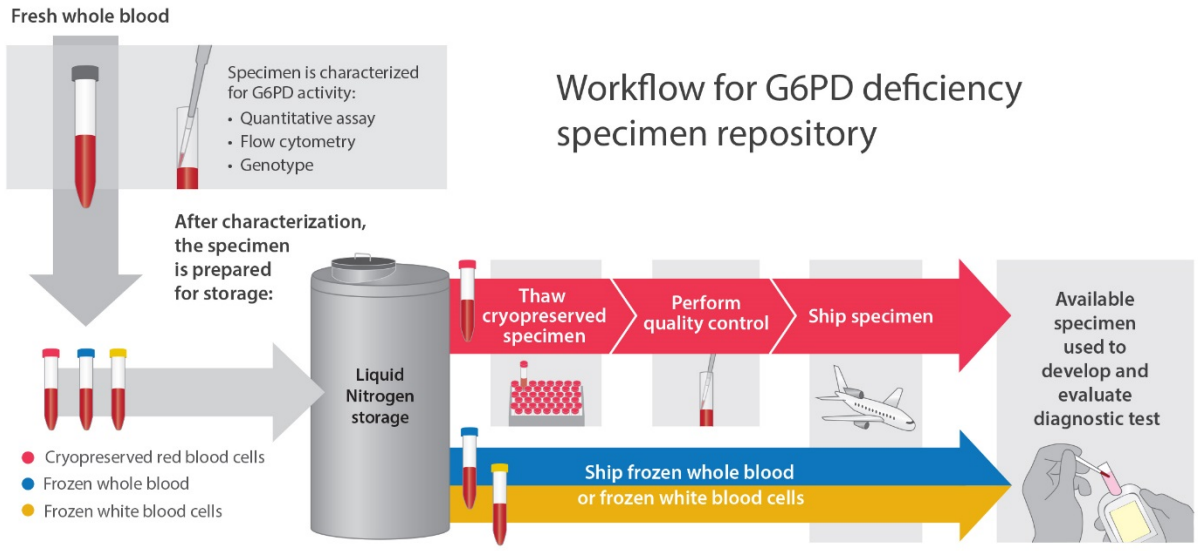


Figure 3. PATH's process for characterizing, preserving, and distributing blood samples for evaluating G6PD point-of-care diagnostic tests.

[b] Red blood cells have no nucleus and thus no DNA.

Value for money provided by the specimen repository

The PATH central repository for blood specimens characterized for G6PD activity offers value measured in several ways for developers, donors, and the larger community working to eliminate malaria.

Value to test developers

PATH will provide two types of services to diagnostic developers. First, PATH can provide specimen panels to developers for evaluation of their prototypes in-house. Second, test developers may send their prototypes to PATH for evaluation—this is a current activity. The cost of acquiring and characterizing 100 specimens for G6PD activity (quantitative and qualitative tests), flow cytometry, and DNA sequencing has been calculated at $71,109 ($711/sample), with the samples obtained at a rate of no more than 15 per week. The overall value delivered by the repository increases with each additional developer served. When PATH prospectively performs this service and distributes the specimens to, for example, three commercial developers, the cost savings to the project is approximately $45,000 per developer, with the additional benefit of a reduced timeline, and associate costs, of six weeks to several months for the developers, had they obtained specimens independently.

Value to donors and the community

As shown in Figure 4, the value of each specimen to the development community can be represented in terms of the value added per specimen, with the value of each specimen increasing as it is distributed to more developers. The return on the initial investment becomes positive with just two participating developers, and at higher numbers of developers the return is many times the initial investment. For example, a specimen that is characterized by flow cytometry and DNA sequencing costs PATH—or ultimately, the funder—$711 to acquire and preserve. The savings are approximately $600 per specimen when PATH gives aliquots to ten developers rather than giving newly sourced specimens to each developer. If ten developers each receive 100 specimens, this represents a realized value of $600,000 and up to several months of each developer's timeline.

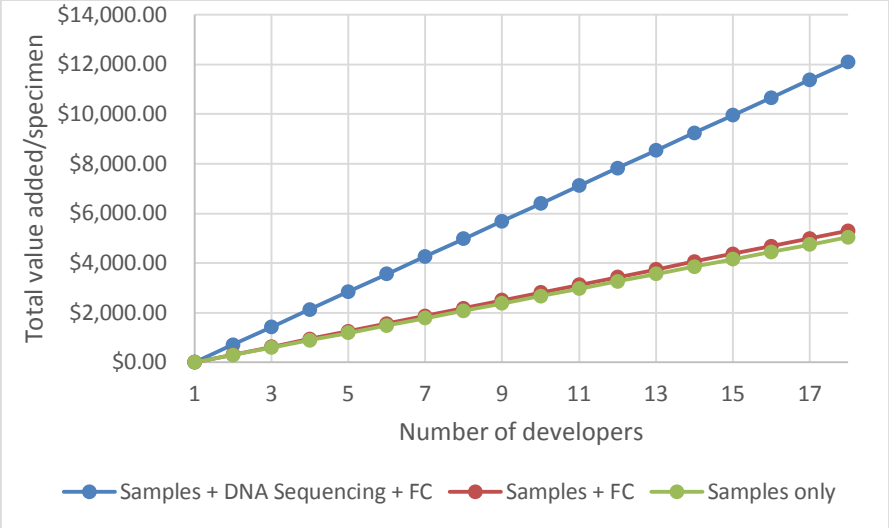


Figure 4. The specimen repository increases the value of the initial donor investment. The value added per specimen is calculated as the difference between the cost of providing unique characterized specimen panels to each developer on demand and the cost of prospectively preserving those panels and distributing them to multiple developers. FC = Flow cytometry

The G6PD specimen repository is not a simple specimen bank, but a means to provide a unique resource that will save both time and money and will benefit the larger community. Saving time means that development of an appropriate quantitative POC G6PD test will be accelerated, the test will arrive more quickly on the market, and the impact on *P. vivax* malaria will begin sooner—contributing to the donors' larger goal of malaria elimination.

The specimens in the repository will be made available to all developers who produce tests that meet the target product profile for a POC G6PD test that supports malaria radical cure. In this manner, global access to donor investments in the repository is ensured.

Acknowledgments

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