



Advances in HLH Testing

Primary hemophagocytic lymphohistiocytosis (HLH) is a potentially life-threatening disorder characterized by overwhelming hyperinflammation. Primary HLH is caused by mutations in genes that are generally important for cytotoxic lymphocyte function (NK cells and T cells). Over the last few decades, research on the genetics and pathophysiology of HLH has greatly improved our understanding of this condition. Consequently, newer clinical laboratory assays have evolved that offer time-sensitive and reliable diagnostic screening for genetic HLH disorders (Table 1). Direct measurement of several proteins, which are defective or deficient in patients with genetic forms of HLH, can be performed. Measurement of perforin, SAP, and XIAP can screen patients for Familial HLH Type 2 due to *PRF1* mutations, X-linked Lymphoproliferative Disease Type 1 (XLP1), and XLP2, respectively. Evaluation of NK cell degranulation can be performed with a CD107a test to screen for several genetic HLH diseases which are associated with abnormal degranulation including Familial HLH Types 3-5 (due to *UNC13D*, *STX11*, or *STXBP2* mutations, respectively), Griscelli Syndrome (due to mutations in *RAB27A*), and Chediak-Higashi Syndrome (due to mutations in *LYST*). These rapid screening tests can help physicians identify immunologic defects that suggest a diagnosis of a genetic form of HLH.

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Disease	Gene	Protein	Rapid Screening Test
Familial Hemophagocytic Lymphohistiocytosis 2	<i>PRF1</i>	Perforin	Perforin Expression
Familial Hemophagocytic Lymphohistiocytosis 3	<i>UNC13D</i>	Munc13-4	CD107a
Familial Hemophagocytic Lymphohistiocytosis 4	<i>STX11</i>	Syntaxin 11	CD107a
Familial Hemophagocytic Lymphohistiocytosis 5	<i>STXBP2</i>	Munc18-2	CD107a
X-Linked Lymphoproliferative Disease Type 1	<i>SH2D1A</i>	SAP	SAP Expression
X-Linked Lymphoproliferative Disease Type 2	<i>XIAP/BIRC4</i>	XIAP	XIAP Expression
Griscelli Syndrome	<i>RAB27A</i>	Rab27a	CD107a
Chediak-Higashi Syndrome	<i>LYST</i>	LYST	CD107a
Hermansky-Pudlak Syndrome Type 2	<i>AP3B1</i>	AP3	CD107a

Table 1. Genetic diseases associated with HLH and rapid screening tests.

In this issue, we'll take a closer look at some of the screening tests that can help clinicians quickly screen patients for genetic HLH disorders while waiting for a confirmatory genetic workup.

NK (Natural Killer Cell) Function Assay

This test has historically been used to help make a diagnosis of HLH. This test provides a measure of the whole process of NK-cell cytotoxicity, including target cell recognition, effector cell activation, transport and exocytosis of lytic granule contents, and target cell death. However, its diagnostic accuracy with respect to screening for genetic HLH has recently been compared to alternative testing using perforin staining and CD107a degranulation testing. Rubin et al found that perforin plus CD107a testing performed better than the traditional chromium release NK cell function test with regard to accurately screening patients for genetic HLH caused by mutations in *PRF1* or the degranulation genes.

Perforin and Granzyme B Assay by Flow Cytometry

This test measures intracytoplasmic perforin in cytotoxic lymphocytes (NK cells and CD8+ T cells). Lytic granules inside cytotoxic lymphocytes, traffic to the contact site between the effector cell and target cell, dock and fuse with the plasma membrane. They then release their contents, which include perforin and granzymes and other substances, into the contact site between the two cells. Perforin oligomerizes and forms pores in the presence of calcium on the surface membrane of the target cell, allowing the entry of granzymes which then activate a cascade that eventually leads to apoptosis of the target cell.

In this assay, patient cells are stained with antibodies against CD45, TCR α/β , CD8 and CD56, then fixed, permeabilized and stained with antibodies against perforin and granzyme B. Samples are analyzed by flow cytometry, and perforin and granzyme B expression are measured in the CD8+ T cells and NK cells. See Figure 1.

A reduction or absence of perforin expression is observed in almost all patients with biallelic mutations in *PRF1*. In our experience, this test has a sensitivity of 96.6% when using a ROC-derived optimal diagnostic threshold.

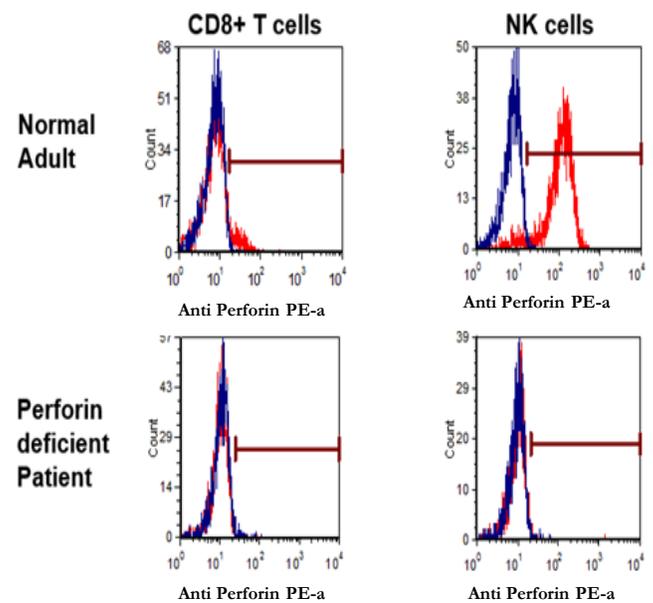


Figure 1. The top histograms show perforin expression (peaks in red) from a normal adult in both CD8+ T cells and NK cells. The two bottom histograms show lack of perforin expression for these cells in a patient with biallelic *PRF1* mutations.



CD107a Assay by Flow Cytometry

CD107a, also known as LAMP-1 (Lysosome-associated membrane protein-1), is normally expressed within the granule membranes of cytotoxic cells. Very little is found on the outer membrane of cytotoxic cells at rest. When a cytotoxic cell releases its granule contents, CD107a will be transiently expressed on the effector cell surface during this process.

Mononuclear cells from patients are stimulated with K562 target cells which induce degranulation. Fluorochrome-conjugated antibodies against CD107a are added during the stimulation. After an incubation period, the cells are additionally surface stained with markers that identify NK and cytotoxic CD8+ T cells. Surface CD107a is then measured using flow cytometry. See Figure 2.

Patients with defects in genes involved in the cytotoxic degranulation pathway (which includes those with biallelic mutations in *UNC13D*, *STXBP2*, *STX11*, *RAB27A*, *LYST*, and *AP3B1*, but not *PRF1*, *SH2D1A* or *XIAP/BIRC4*) have been shown to exhibit decreased NK-cell surface CD107a upregulation upon exposure to target cells. In our experience, the sensitivity of this test to identify these patients is 93.8% using a ROC-derived optimal diagnostic threshold.

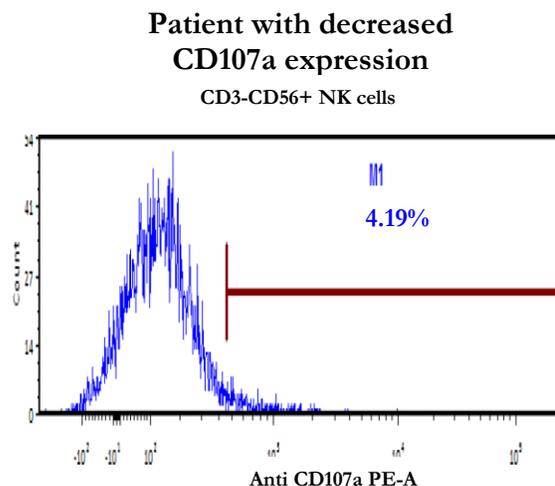
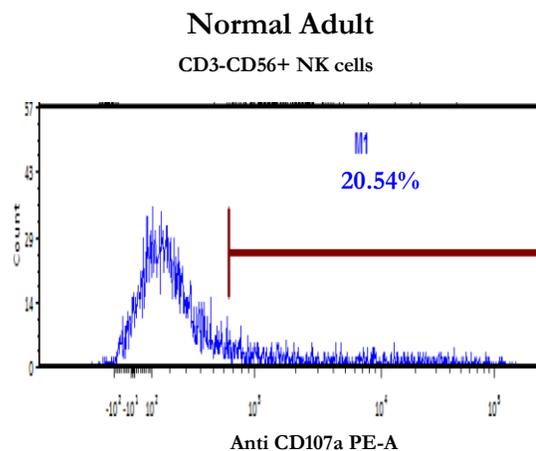


Figure 2. The top histogram represents surface CD107a on NK cells in a normal adult. The reference range is 11-35%. The bottom histogram shows decreased surface CD107a on NK cells from a patient with biallelic *UNC13D* mutations.

SAP Assay by Flow Cytometry

The most common cause of XLP (X-linked Lymphoproliferative Disease) is deficiency of SLAM-Associated Protein (SAP) caused by mutations in the *SH2D1A* gene. XLP is a primary immunodeficiency characterized by an extreme susceptibility to EBV (Epstein-Barr virus) and should always be considered in males with EBV-associated HLH. HLH may develop because of absence of iNKT cells, defective 2B4-mediated cytotoxic lymphocyte cytotoxicity, and defective T-cell reactivation-induced cell death (RICD). Patients may also develop lymphoma, hypogammaglobulinemia, or other more rare problems.

In order to screen for this deficiency, patient lymphocytes are stained with antibodies against CD3, CD8, and CD56, then fixed, permeabilized and stained with an anti-SAP antibody. Samples are analyzed by flow cytometry and SAP expression is measured. See Figure 3.

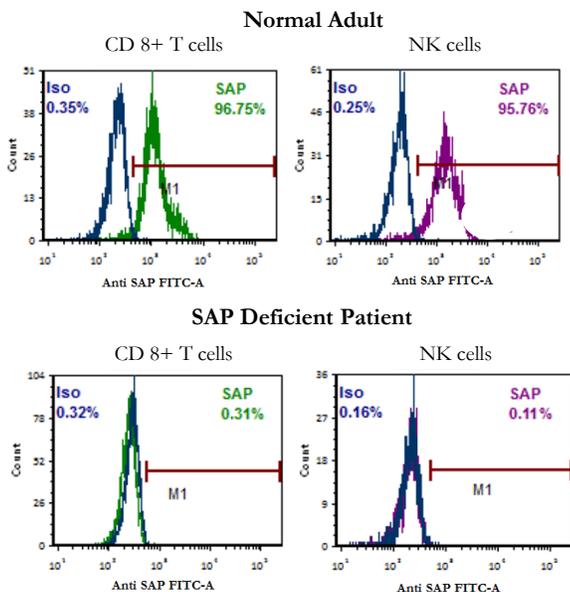


Figure 3. Histograms in the top row show expression of SAP within CD8+ T cells (green peak) and NK cells (purple peak) from a normal control. Histograms in the bottom row demonstrate essentially absent expression of SAP in a patient with a *SH2D1A* mutation.

XIAP Assay by Flow Cytometry

Deficiency of X-linked Inhibitor of Apoptosis Protein (XIAP), caused by mutation in the *XIAP/BIRC4* gene, is the second most common cause of XLP. XLP2 is associated with the development of HLH, recurrent infections, inflammatory bowel disease, and other problems. Although this is an X-linked gene, female carriers may occasionally develop disease, particularly if a high proportion of cells lack XIAP due to skewed X chromosome lyonization.

To identify XIAP deficiency, patient cells are stained with surface markers and then fixed, permeabilized, and stained with a fluorochrome-conjugated antibody against XIAP. Expression of XIAP is measured in lymphocytes, monocytes, and granulocytes. See Figure 4.

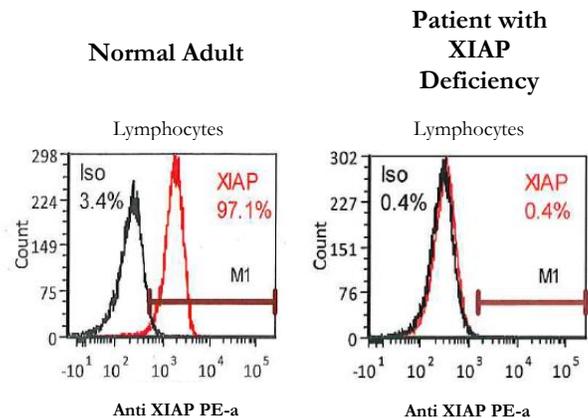
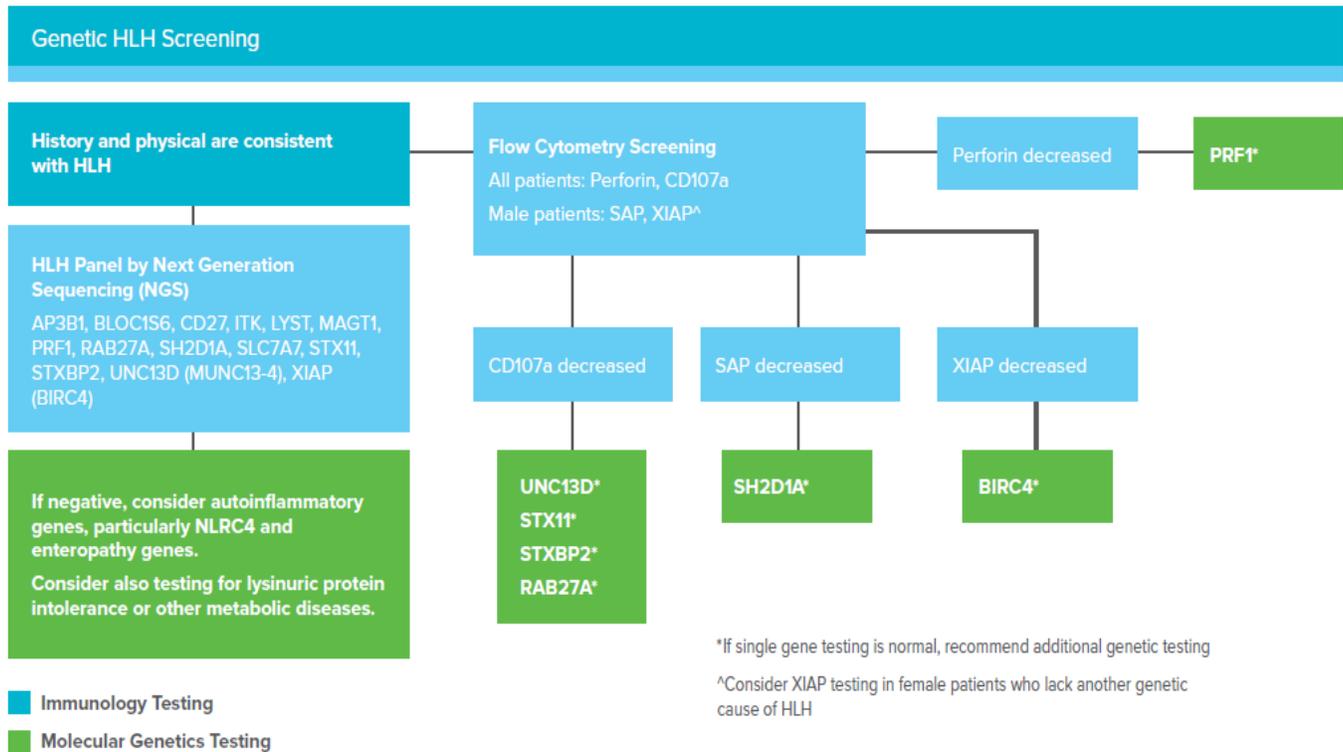


Figure 4. The left histogram shows XIAP staining in lymphocytes (peak in red) from a normal adult. The right histogram is from a patient with a mutation in the *XIAP/BIRC4* gene. Notice the lack of XIAP expression in the lymphocytes.

Need help in determining the most efficient sequence of testing for your patients? Below is an algorithm that was developed by physicians at CCHMC specifically for cases where genetic HLH is suspected. This table is available to view at www.cincinnatichildrens.org/dchi



- If absolute lymphocyte count <300cells/μL, proceed to sequencing in lieu of flow cytometry testing.
- Saliva is preferred method of sample collection in patients with blood cell counts <2000 cells/ μL.

Visit the Diagnostic Center for Heritable Immunodeficiencies at www.cincinnatichildrens.org/dchi or call 513-636-4474.

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