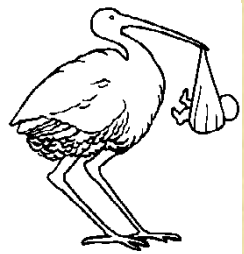
Molecular Testing: Applications in Screening Newborns for Hemoglobinopathies and Galactosemia

Michael Glass, Director (retired)

Newborn Screening Program

Washington State Department of Health

In the Beginning:
Science, November1949



Reprinted from SCIENCE, November 25, 1949, Vol. 110, No. 2865, pages 543–548.

Sickle Cell Anemia, a Molecular Disease

Linus Pauling, Harvey A. Itano, S. J. Singer, and Ibert C. Wells
Gates and Crellin Laboratories of Chemistry,
California Institute of Technology, Pasadena, California

May1972

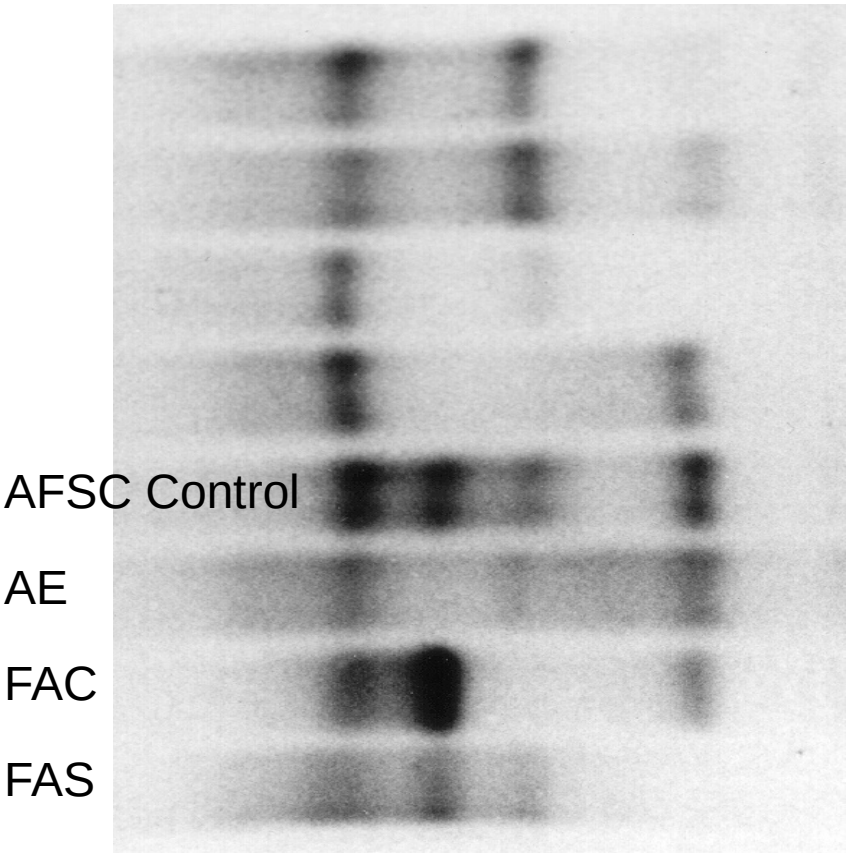
National Sickle Cell Anemia Control Act

Funds and promotes population screening and education

Solubility Test

Alkaline Electrophoresis

(cellulose acetate)



1986

Prophylaxis with Oral Penicillin in Children with Sickle Cell Anemia

- Marilyn H. Gaston, M.D., Joel I. Verter, Ph.D., Gerald Woods, M.D., Charles Pegelow, M.D., John Kelleher, M.D., Gerald Presbury, M.D., Harold Zarkowsky, M.D., Elliott Vichinsky, M.D., Rathi Iyer, M.D., Jeffrey S. Lobel, M.D., Steven Diamond, M.D., C. Tate Holbrook, M.D., Frances M. Gill, M.D., Kim Ritchey, M.D., John M. Falletta, M.D., and For the Prophylactic Penicillin Study Group

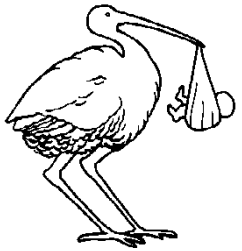
N Engl J Med 1986; 314:1593-1599

June 19, 1986

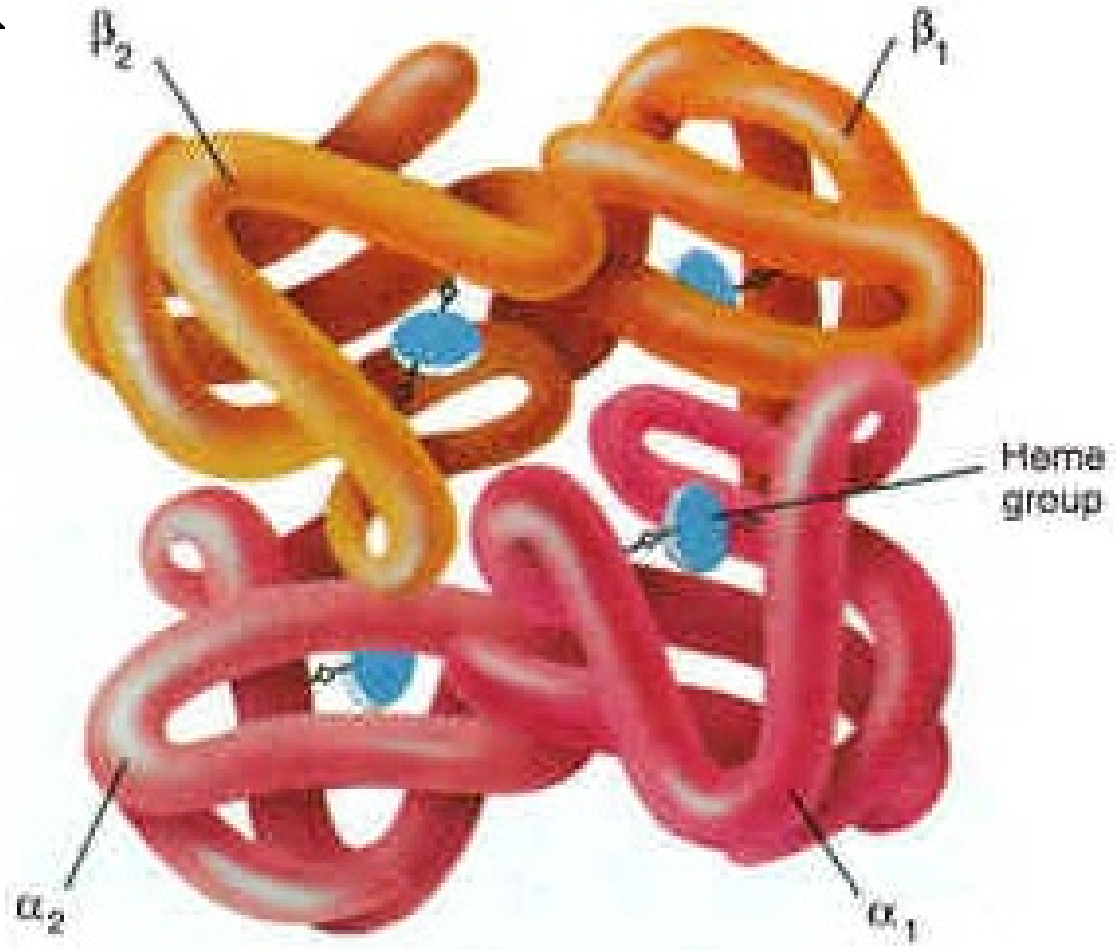
1987

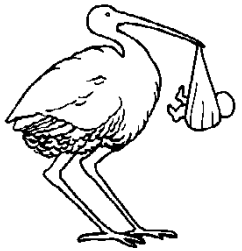
NIH Consensus Conference:

"The benefits of screening are so compelling that universal screening should be provided…"

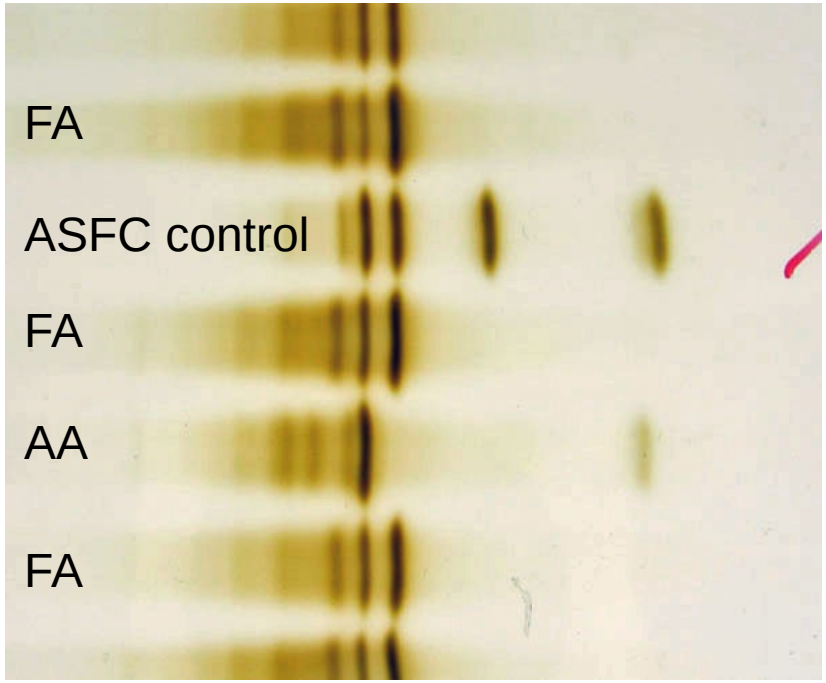


The α,β,λ's of The Hb Molecule





Isoelectric focusing



Hemoglobin

~7500 Asian infants born in WA each year

Genotype	**Prevalence**	**# Infants/year**
FAE (E trait)	1 in 35	200
FEE (homozygous)	1 in 350	20
FE– (E/β^o thalassemia)	1 in 7000	1

Science 1985

RESEARCH ARTICLE

Enzymatic Amplification of β-Globin Genomic Sequences and Restriction Site Analysis for Diagnosis of Sickle Cell Anemia

Randall K. Saiki, Stephen Scharf, Fred Faloona, Kary B. Mullis
Glenn T. Horn, Henry A. Erlich, Norman Arnheim

Recent advances in recombinant DNA technology have made possible the molecular analysis and prenatal diagnosis of several human genetic diseases. Fetal DNA obtained by aminocentesis or chorionic villus sampling can be analyzed by restriction enzyme digestion, with subsequent electrophoresis, Southern transfer, and specific hybridization to cloned gene or oligonucleotide probes. With polymorphic DNA markers linked genetically to a specific disease locus, segregation analysis must be carried out with restriction fragment length polymorphisms (RFLP's) found to be informative by examining DNA from family members (*1*, *2*).

Many of the hemoglobinopathies, however, can be detected by more direct methods in which analysis of the fetus alone is sufficient for diagnosis. For example, the diagnosis of hydrops fetalis

This disease results from homozygosity of the sickle-cell allele (β^S) at the β-globin gene locus. The S allele differs from the wild-type allele (β^A) by substitution of an A in the wild-type to a T at the second position of the sixth codon of the β chain gene, resulting in the replacement of a glutamic acid by a valine in the expressed protein. For the prenatal diagnosis of sickle cell anemia, DNA obtained by amniocentesis or chorionic villus sampling can be treated with a restriction endonuclease (for example, Dde I and Mst II) that recognizes a sequence altered by the β^S mutation (*8*–*11*). This generates β^A- and β^S-specific restriction fragments that can be resolved by Southern transfer and hybridization with a β-globin probe.

We have developed a procedure for the detection of the sickle cell mutation that is very rapid and is at least two

Abstract. *Two new methods were used to establish a rapid and highly sensitive prenatal diagnostic test for sickle cell anemia. The first involves the primer-mediated enzymatic amplification of specific β-globin target sequences in genomic DNA, resulting in the exponential increase (220,000 times) of target DNA copies. In the second technique, the presence of the β^A and β^S alleles is determined by restriction endonuclease digestion of an end-labeled oligonucleotide probe hybridized in solution to the amplified β-globin sequences. The β-globin genotype can be determined in less than 1 day on samples containing significantly less than 1 microgram of genomic DNA.*

lessen the complexity of prenatal diagnosis for sickle cell anemia; they may also be generally applicable to the diagnosis of other genetic diseases and in the use of DNA probes for infectious disease diagnosis.

Sequence amplification by polymerase chain reaction. We use a two-step procedure for determining the β-globin genotype of human genomic DNA samples. First, a small portion of the β-globin gene sequence spanning the polymorphic Dde I restriction site diagnostic of the β^A allele is amplified. Next, the presence or absence of the Dde I restriction site in the amplified DNA sample is determined by solution hybridization with an end-labeled complementary oligomer followed by restriction endonuclease digestion, electrophoresis, and autoradiography.

The β-globin gene segment was amplified by the polymerase chain reaction (PCR) procedure of Mullis and Faloona (*12*) in which we used two 20-base oligonucleotide primers that flank the region to be amplified. One primer, PC04, is complementary to the (+)-strand and the other, PC03, is complementary to the (−)-strand (Fig. 1). The annealing of PC04 to the (+)-strand of denatured genomic DNA followed by extension with the Klenow fragment of *Escherichia coli* DNA polymerase I and deoxynucleotide triphosphates results in the synthesis of a (−)-strand fragment containing the target sequence. At the same time, a similar reaction occurs with PC03, creating a new (+)-strand. Since these newly synthesized DNA strands are themselves template for the PCR primers, repeated cycles of denaturation, primer annealing, and extension result in the exponential accumulation of the 110–base pair region defined by the primers.

An example of the degree of specific gene amplification achieved by the PCR method is shown in Fig. 2A. Samples of DNA (1 μg) were amplified for 20 cycles and a fraction of each sample, equivalent to 36 ng of the original DNA, was subjected to alkaline gel electrophoresis and

Kary Mullis

PCR Video

Human Genetics 1987

Hum Genet (1987) 75:213–216

Human Genetics

DNA microextraction from dried blood spots on filter paper blotters: potential applications to newborn screening

Edward R.B. McCabe[1,2*], Shu-Zhen Huang[1,3], William K. Seltzer[1,2], and Martha L. Law[1,4]

[1]Department of Pediatrics and [2]Department of Biochemistry, Biophysics, and Genetics, University of Colorado School of Medicine, Denver, CO 80262, USA
[3]Laboratory of Medical Genetics, Shanghai Children's Hospital, Shanghai, Peoples Republic of China
[4]Eleanor Roosevelt Institute for Cancer Research, Denver, CO 80262, USA

Summary. Microextraction of DNA from dried blood specimens would ease specimen transport to centralized laboratory facilities for recombinant DNA diagnosis in the same manner as use of dried blood spots allowed the broad application of screening tests to newborn populations. A method is described which reproducibly yields 0.5 µg DNA from the dried equivalent of 50 µl whole blood. Though DNA yields decreased with storage of dried specimens at room temperature, good-quality DNA was still obtained. Sufficient DNA was routinely obtained for Southern blot analysis using repetitive and unique sequences. This microextraction procedure will allow immediate application of molecular genetic technology to direct newborn screening follow-up of disorders amenable to DNA diagnosis, such as sickle cell anemia, and may eventually permit primary DNA screening for specific mutations.

Introduction

Newborn screening efforts were facilitated by the use of dried blood spots on filter paper blotters, since these blotters simplified shipment of specimens and allowed centralization of laboratory facilities (Guthrie 1980). These dried blood samples have been used for various tests including those based on screening for red blood cell proteins, as in galactosemia or the hemoglobinopathies, for hormonal measurements, as in hypothyroidism, and for metabolite accumulation, as in phenyl-

Methods

EDTA-anticoagulated fresh whole blood specimens were drawn, white blood cells prepared, and DNA was extracted using modifications of previously described methods (Kan et al. 1977; Ponez et al. 1982). Dried blood specimens were prepared on Schleicher and Schuell newborn screening blotters or Whatman 3 MM filter paper. Measured aliquots of whole blood were spotted directly or anticoagulated with EDTA before spotting. Dried blood specimens were stored at room temperature.

After several methods of extraction had been investigated, including direct total proteolysis of the dried blood specimen similar to that described for specimens dried on cotton cloth (Gill et al. 1985), we settled on the following procedure involving white blood cell rehydration, since it gave the highest reproducible yields of DNA. A dried blood spot on filter paper equivalent to 50 µl whole blood was shredded or minced and placed in a 10-ml plastic tube. Three milliliters 0.85% NaCl were added and allowed to stand at room temperature for 1 h with occasional gentle shaking. The 3-ml fluid layer was then pipetted off, leaving the paper behind. A 1.5-ml aliquot was placed into a 1.5-ml Eppendorf tube and centrifuged, the white blood cell pellet retained, and the supernatant discarded. This procedure was repeated with the remaining 1.5-ml aliquot in the same Eppendorf tube. The paper was washed with 1.5 ml 0.85% NaCl and mixed gently for 5 min. The liquid was added to the same Eppendorf tube containing the white blood cell pellet and centrifuged. The combined pellet

Science1988

Science 29 January 1988:
Vol. 239 no. 4839 pp. 487-491
DOI: 10.1126/science.2448875

Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase

RK Saiki, et. al

Cetus Corporation, Department of Human Genetics, Emeryville, CA 94608.

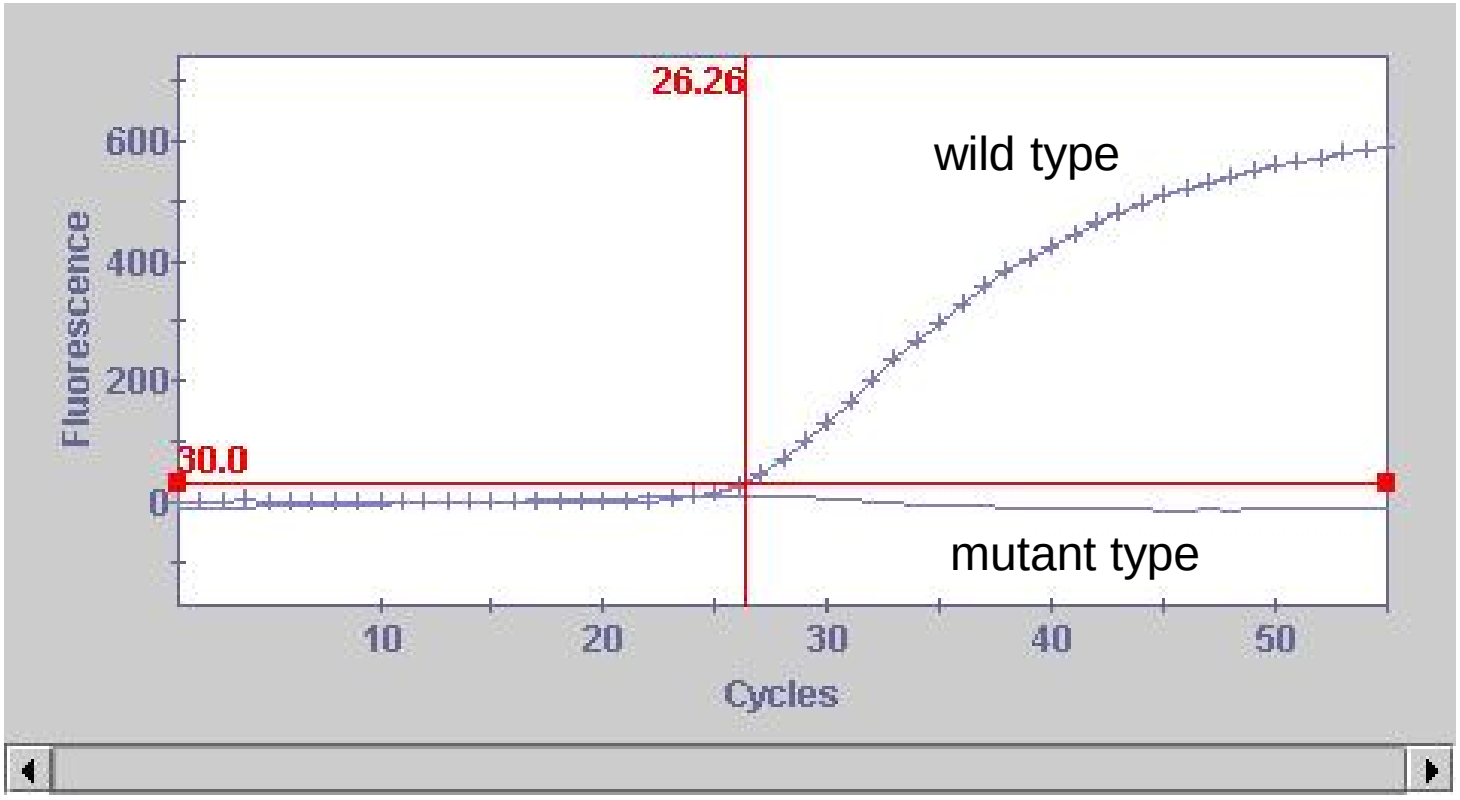
1994

Washington adds DNA/PCR to the routine screening algorithm for hemoglobinopathies.

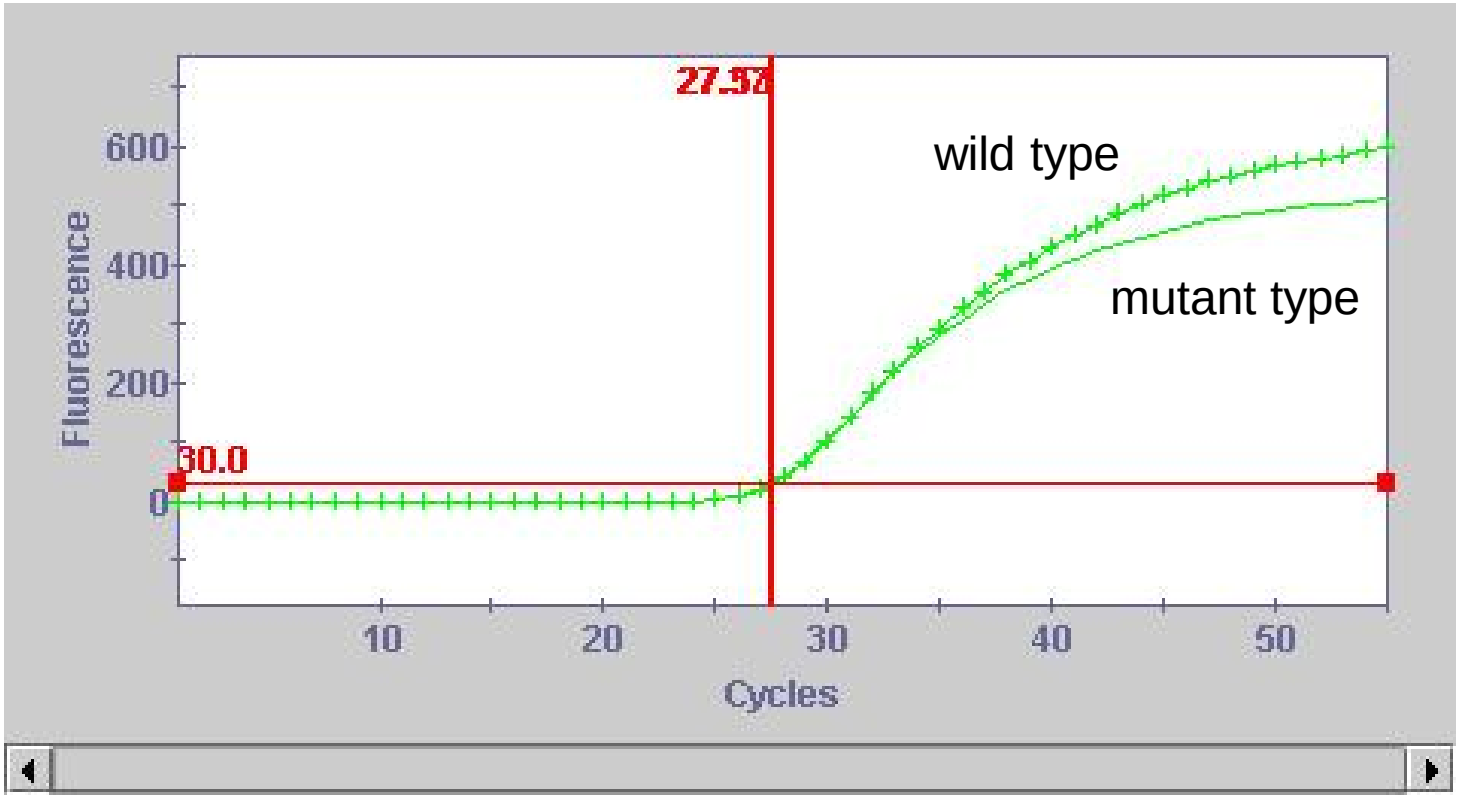
DNA/PCR Methods

Method	Assay Time
PCR restriction enzyme analysis	3 days
Real Time PCR (SmartCycler® II)	3 hours

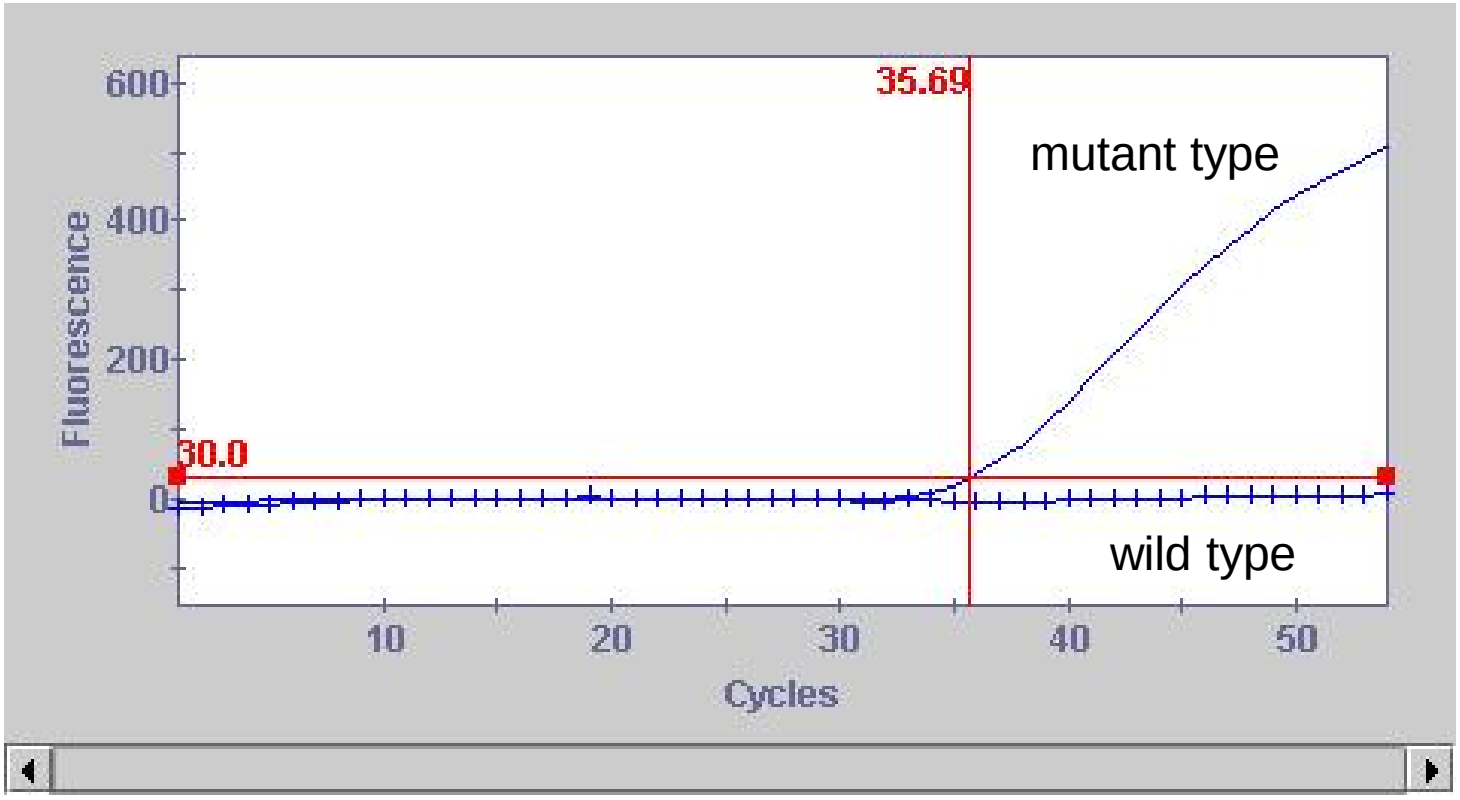
Normal


26.26
wild type
600
400
200
Fluorescence
30.0
0
mutant type
10
20
30
40
50
Cycles

Heterozygote



Homozygote


600
400
200
0
Fluorescence
35.69
30.0
mutant type
wild type
10
20
30
40
50
Cycles

Hemoglobin

Screening Protocol:

IEF → HPLC → DNA/PCR

Mutations:

β-globin: S, E, C,

(*α-globin: Constant Spring*)

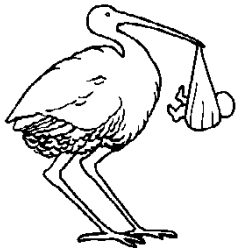
Hemoglobin

Phenotype	**DNA Probe**
FS	S → SS vs S/βº thalassemia
FE	E → EE vs E/βº thalassemia
FC	C → CC vs C/βº thalassemia
High Bart's	*Constant Spring – Hb H vs Hb H/CS*

Limitation/Caution

DNA is helpful but not perfect:

No response from the HbA probe can be due to a thalassemia deletion that encompasses the site.

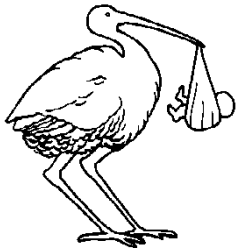


Galactosemia

Washington was the last to add (2004)

Lessons learned from others:

- **Deadly disorder**
- **Galt enzyme labile in heat and humidity**
- **Duarte variant modifies severity**



Galactosemia

Screening Protocol:

GALT enzyme → Total galactose → DNA/PCR

Mutations:

Q188R, S135L, K285N,

& N314D (Duarte variant)

Galactosemia

frequency of classic alleles

Mutation	% of total
Q188R	54.1%
S135L	8.4%
K285N	4.8%
L195P	2.6%
Y209C	1.2%
F171S	1.0%
Private DNA Sequencing	18.0%
Unknown	9.9%

of 250 patients in the U.S.
(from genetests.org)

Galactosemia

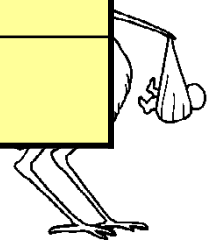
GALT (Units/gHb)	First Specimen	Second Specimen	
		≥ 2.9	< 2.9
≥ 2.9	NO	NO	YES
< 2.9	YES	NO	NO*

* DNA analysis should already have been done on first specimen

Galactosemia

DNA results on 41 infants

Genotype	# Infants	% of total
G/G	1	2%
G/?	9	22%
D/G	13	32%
D/D	0	0%
D/?	7	17%
None	11	27%



Galactosemia

for G/? genotype

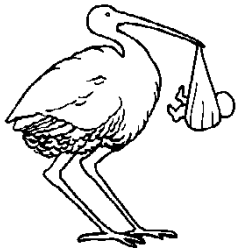
# Infants	Outcome
4	true positive (disease)
4	false positive (carrier)
1	Likely carrier Mom refused dx

Limitations/Caution

DNA is helpful but not perfect:

A D/G finding does not always mean a DG phenotype:

1. They could be on the same chromosome
2. There may be another severe mutation that is not on the screening panel.



Other Approaches

- DNA Bead Technology
- LightCycler
- Microarrays
- Sequencing
- etc

Take home messages

- Second tier targeted DNA can provide valuable information to guide follow-up for hemoglobins, galactosemia and other conditions
- However, there are important limitations that must be taken into account.
- DNA is not always the panacea we wish it was!