Molecular Testing: Applications in Screening Newborns for Hemoglobinopathies and Galactosemia

Michael Glass, Director (retired)
Newborn Screening Program
Washington State Department of Health
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
National Sickle Cell Anemia Control Act

Funds and promotes population screening and education
Solubility Test
Alkaline Electrophoresis
(cellulose acetate)
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


June 19, 1986
NIH Consensus Conference:

“The benefits of screening are so compelling that universal screening should be provided….”
The $\alpha, \beta, \lambda$’s of
The Hb Molecule
Isoelectric focusing
Hemoglobin

~7500 Asian infants born in WA each year

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Prevalence</th>
<th># Infants/year</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAE (E trait)</td>
<td>1 in 35</td>
<td>200</td>
</tr>
<tr>
<td>FEE (homozygous)</td>
<td>1 in 350</td>
<td>20</td>
</tr>
<tr>
<td>FE– (E/$\beta^o$ thalassemia)</td>
<td>1 in 7000</td>
<td>1</td>
</tr>
</tbody>
</table>
Enzymatic Amplification of β-Globin Genomic Sequences and Restriction Site Analysis for Diagnosis of Sickle Cell Anemia

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

Recent advances in recombinant DNA technology have made possible the molecular analysis and prenatal diagnosis of several human genetic diseases. Fetal DNA obtained by amniocentesis 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

This disease results from homozygosity of the sickle-cell allele (βS) at the β-globin gene locus. The S allele differs from the wild-type allele (βA) 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 in the expressed protein. For the prenatal diagnosis of sickle cell anemia, DNA ob-

Sequence amplification by polymerase chain reaction. We use a two-step procedure for determining the β-globin genotype of human genome that flank the region of β-globin gene sequence spanning the polymorphic Dde I restriction site diagnostic of the β allele is amplified. Next, the presence of 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 Falouoa (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 deoxynucleotides 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
DNA microextraction from dried blood spots on filter paper blotters: potential applications to newborn screening

Edward R. B. McCabe1,2,*, Shu-Zhen Huang1,3, William K. Seltzer1,2, and Martha L. Law1,4

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3Laboratory of Medical Genetics, Shanghai Children's Hospital, Shanghai, Peoples Republic of China
4Eleanor 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.

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

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

RK Saiki, et. al

Cetus Corporation, Department of Human Genetics, Emeryville, CA 94608.
Washington adds DNA/PCR to the routine screening algorithm for hemoglobinopathies.
## DNA/PCR Methods

<table>
<thead>
<tr>
<th>Method</th>
<th>Assay Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR restriction enzyme analysis</td>
<td>3 days</td>
</tr>
<tr>
<td>Real Time PCR (SmartCycler® II)</td>
<td>3 hours</td>
</tr>
</tbody>
</table>
Normal

wild type

mutant type
Heterozygote

wild type

mutant type
Homozygote

mutant type

wild type
Hemoglobin

Screening Protocol:
IEF → HPLC → DNA/PCR

Mutations:
β-globin: S, E, C,
(α-globin: Constant Spring)
**Hemoglobin**

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>DNA Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>FS</td>
<td>S → SS vs S/β° thalassemia</td>
</tr>
<tr>
<td>FE</td>
<td>E → EE vs E/β° thalassemia</td>
</tr>
<tr>
<td>FC</td>
<td>C → CC vs C/β° thalassemia</td>
</tr>
<tr>
<td><em>High Bart’s</em></td>
<td><em>Constant Spring – Hb H vs Hb H/CS</em></td>
</tr>
</tbody>
</table>
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

<table>
<thead>
<tr>
<th>Mutation</th>
<th>% of total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q188R</td>
<td>54.1%</td>
</tr>
<tr>
<td>S135L</td>
<td>8.4%</td>
</tr>
<tr>
<td>K285N</td>
<td>4.8%</td>
</tr>
<tr>
<td>L195P</td>
<td>2.6%</td>
</tr>
<tr>
<td>Y209C</td>
<td>1.2%</td>
</tr>
<tr>
<td>F171S</td>
<td>1.0%</td>
</tr>
<tr>
<td>Private DNA Sequencing</td>
<td>18.0%</td>
</tr>
<tr>
<td>Unknown</td>
<td>9.9%</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>GALT (Units/gHb)</th>
<th>First Specimen</th>
<th>Second Specimen</th>
</tr>
</thead>
<tbody>
<tr>
<td>≥ 2.9</td>
<td>NO</td>
<td>NO</td>
</tr>
<tr>
<td>&lt; 2.9</td>
<td>YES</td>
<td>NO</td>
</tr>
</tbody>
</table>

* DNA analysis should already have been done on first specimen

* DNA analysis should already have been done on first specimen
Galactosemia

DNA results on 41 infants

<table>
<thead>
<tr>
<th>Genotype</th>
<th># Infants</th>
<th>% of total</th>
</tr>
</thead>
<tbody>
<tr>
<td>G/G</td>
<td>1</td>
<td>2%</td>
</tr>
<tr>
<td>G/?</td>
<td>9</td>
<td>22%</td>
</tr>
<tr>
<td>D/G</td>
<td>13</td>
<td>32%</td>
</tr>
<tr>
<td>D/D</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>D/?</td>
<td>7</td>
<td>17%</td>
</tr>
<tr>
<td>None</td>
<td>11</td>
<td>27%</td>
</tr>
</tbody>
</table>
Galactosemia

for G/? genotype

<table>
<thead>
<tr>
<th># Infants</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>true positive (disease)</td>
</tr>
<tr>
<td>4</td>
<td>false positive (carrier)</td>
</tr>
<tr>
<td>1</td>
<td>Likely carrier</td>
</tr>
<tr>
<td></td>
<td>Mom refused dx</td>
</tr>
</tbody>
</table>
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!