In conclusion, we present a rate kinetic method for the analysis of plasma Hb that is an adaptation of previous assays based on endpoint measurement (8, 9). The current assay is not subject to ascorbate interference or transient absorbance changes inherent in the endpoint assays that use chlorpromazine (8, 9).

References

Fig. 3. Inter- and intratube variability of plasma Hb concentration in a blood specimen collected into separate 1-mL (C) and 10-mL Disposable Products heparin tubes (A, B). The 5-mL EDTA Exetainer tubes (D), and 4-mL heparin (E) and EDTA (F) Vacutte tubes Also shown is the result of this blood collected into a syringe heparinized with a 1000 IU/mL heparin solution (G) and dispensed as 10 mL aliquots into untreated plastic tubes

In plasma samples collected from 17 healthy laboratory staff into heparin Vacutte tubes (7 men, 10 women; ages 20 to 54 years), the Hb concentrations ranged from 6 to 35 mg/L. Consequently, we propose that the normal plasma Hb concentration for our method is <50 mg/L, in agreement with other methods (1). In normal urine samples, hemoglobin was not detected.

Fast, Manual, Nonradioactive Method for DNA Sequencing

Brigitte Debuire,1 Alie Chabl, and Nicole Fresy

We describe a protocol that allows nonradioactive detection of sequencing products after manual, direct, solid-phase sequencing of polymerase chain reaction-amplified DNA. The amplified DNA fragment to be studied is biotinylated at the 5' end of one of the two oligonucleotide primers used for amplification, allowing coupling to streptavidin-coated magnetic beads. The immobilized double-stranded DNA is then separated into single strands by alkaline treatment. A 5'-biotinylated sequencing primer is used after saturating with a biotin solution any possible remaining affinity sites on the streptavidin-coated magnetic beads. Sequencing is performed by using T7 DNA polymerase, and the sequencing products are electrophoresed in denaturing polyacrylamide sequencing gel. After transfer of the products to a nylon membrane, the sequencing pattern is revealed by chemiluminescence. Biotinylated alkaline phosphatase is bound to the 5' end of the sequencing primer via a streptavidin bridge and catalyzes the reaction by cleaving a phosphate group from a chemiluminescent substrate. The emitted photons are detected by exposing the membrane to x-ray film. This method is simple, rapid, and consistently successful and reproducible.

Indexing Terms: polymerase chain reaction · biotin-streptavidin interaction · chemiluminescence · enzymatic methods · electrophoresis, polyacrylamide gel

CLIN. CHEM. 39/8, 1682–1685 (1993)

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Received December 3, 1992; accepted March 2, 1993.

1682 CLINICAL CHEMISTRY, Vol. 39, No. 8, 1993
In the past decade, application of molecular biology techniques to the diagnosis of malignancies or hereditary diseases has gained a growing interest (1). Frequently, there is a need to determine human DNA sequences in clinical laboratories. In many such laboratories, automatic sequencing systems are often not affordable, and handling of radioisotopes for manual sequencing is also a problem. In addition, cloning the DNA before sequencing is time consuming and presents another important difficulty for clinical laboratories. The recent progress in direct solid-phase sequencing (2, 3) and chemiluminescent detection of DNA (4–6) has allowed us to adapt a simple, manual, rapid, nonradioactive solid-phase approach to DNA sequencing. It was tested after polymerase chain reaction (PCR) amplification (7) of a cDNA fragment encompassing the bcr-abl junction in a chronic myeloid leukemia (CML) patient before bone marrow transplantation. In CML, the fused bcr-abl gene results from the Philadelphia chromosome translocation t(9;22)(q34;q11) (8). PCR is now used extensively to detect residual bcr/abl chimeric transcripts in CML patients who have received a bone marrow transplant (9, 10). Here, we describe our use of the well-known bcr/abl sequence (11) as a reference to demonstrate a DNA sequencing method that can be widely used in the molecular diagnosis of malignant or hereditary diseases.

Materials and Methods

PCR amplification of the bcr-abl junction. RNA was extracted from nucleated cells of peripheral blood from a CML patient as described elsewhere (12). After reverse transcription with the oligonucleotide 5'TGTGATTATAGCTAAGACCCGGAG 3' (primer A), the specific bcr-abl junction was amplified by a two-step PCR procedure in which products of the first amplification were reamplified by using nested primers (13). PCR amplifications were carried out with a Hybaid thermocycler (Cera Labo-France, Aubervilliers, France) with thermostable DNA polymerase (Replitherm; Epicentre Technologies, Madison, WI). The first round of amplification consisted of 30 cycles (each cycle: 98°C for 3 min, 65°C for 2 min, and 72°C for 3 min) and involved a combination of primer A and oligonucleotide 5'GAAAGGTGGTCAGAAGCTTCTCC 3' (primer B).

Part (2.5%) of the first PCR product was used for a second amplification procedure with nested primers: the 5'-biotinylated primer C (oligonucleotide 5'biot-TCCACTGGGCCACAAAAATGCATACGT 3') and the nonbiotinylated primer D (oligonucleotide 5'GTGAAACTCCAGA-CTGGTCACAGC 3'). This second round consisted of 30 cycles (each cycle: 93°C for 2 min, 65°C for 1 min, and 72°C for 2 min). The combination C/D brackets a cDNA fragment of 198 bp. The final PCR volume was 100 μL; 5 μL was removed for testing by electrophoresis on a 2.5% agarose gel; the remaining 95 μL was used for sequencing.

Direct solid-phase sequencing with magnetic beads. Magnetic beads (Dynabeads M-280, Streptavidin; Dynal AS, Oslo, Norway), 80 μL, were washed twice with 1 mL of a solution of NaCl (1 mol/L), Tris·HCl (10 mmol/L, pH 7.5), and EDTA (1 mmol/L) in a 1.5-mL siliconized Eppendorf tube. The biotinylated PCR product was added and allowed to bind for 20 min. The beads were then separated by a magnet and washed with 1 mL of Tris-EDTA buffer (Tris·HCl, 10 mmol/L, pH 7.5, plus EDTA, 1 mmol/L). The immobilized double-stranded DNA was converted into single-stranded form by incubation for 10 min at room temperature with 150 μL of NaOH (0.2 mol/L). The magnetic beads were separated and washed twice with 1 mL of Tris-EDTA buffer. Because a 5'-biotinylated primer is used for sequencing, we introduced here a step to saturate the possible remaining affinity sites on the streptavidin-coated magnetic beads. We added 100 μL of a 2-μmol/L solution of biotin in TE buffer, let this stand for 5 min at room temperature, and then washed the magnetic beads twice with 1 mL of Tris-EDTA buffer. For sequencing we used T7 DNA polymerase (T7 sequencing kit; Pharmacia, Uppsala, Sweden) and 5'-biotinylated primer D, as follows: The magnetic beads were resuspended in 10 μL of water, then 2 μL of annealing buffer and 2 μL (10 ng) of sequencing primer were added. The mixture was incubated for 10 min at 65°C and the temperature dropped very slowly to room temperature. We then added 4 μL of water and 2 μL (3 U) of T7 DNA polymerase. Four 5-μL aliquots of this mixture were added to 0.5-mL siliconized Eppendorf tubes containing 2.5 μL of the deoxy/dideoxynucleotide mixes A, G, C, and T and incubated at 37°C for 5 min. After adding 100 μL of water, we separated the magnetic beads by a magnet and incubated the beads with 4 μL of stop solution for 10 min at 65°C. We then froze the beads at ~20°C, added 4 μL of water, heated the samples at 95°C for 2 min, and cooled them in ice before loading them onto a denaturing 8% polyacrylamide sequencing gel.

Chemiluminescent detection of sequencing products. After removing the siliconized upper glass plate, we soaked the gel adhering to the untreated plate in buffer containing, per liter, 45 mmol of Tris-borate and 1 mmol of EDTA; we then transferred the gel to Whatman filter paper 3 MM CHR (Whatman Laboratory Div., Maidstone, UK). A wet Immobilon-S membrane was applied to the gel surface, and the DNA was transferred for 2 h according to the manufacturer’s recommendations (Millipore, Molsheim, France), then cross-linked by exposure to ultraviolet radiation. Biotinylated alkaline phosphatase was bound to the 5' end of the sequencing primer via a streptavidin bridge and catalyzed the light reaction by cleaving a phosphate group from a chemiluminescent substrate, Lumigen PPD reagent (Plex™ luminescent kits; Millipore). The emitted photons were detected by exposing the membrane to x-ray film (Kodak X-OMAT AR; Eastman Kodak, Rochester, NY) for 10 min at room temperature. The experimental conditions required for each step are reported in Table 1.

Results and Discussion

The results in Figure 1A show amplification of a 198-bp fragment representing the bcr-abl junction in a
Table 1. Experimental Conditions for Chemiluminescent Detection of DNA Sequencing Products

<table>
<thead>
<tr>
<th>Reagent or procedure</th>
<th>Time, min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubation steps</td>
<td></td>
</tr>
<tr>
<td>Blocking solution</td>
<td>15</td>
</tr>
<tr>
<td>Streptavidin solution</td>
<td>5</td>
</tr>
<tr>
<td>Wash solution</td>
<td>5, 5, 10</td>
</tr>
<tr>
<td>Biotinylated alkaline phosphatase solution</td>
<td>5</td>
</tr>
<tr>
<td>Wash solution</td>
<td>5, 5, 10</td>
</tr>
<tr>
<td>Lumigen PPD reagent</td>
<td>5</td>
</tr>
<tr>
<td>Detection</td>
<td></td>
</tr>
<tr>
<td>Incubation in the dark</td>
<td>120</td>
</tr>
<tr>
<td>Exposure to x-ray film</td>
<td>10</td>
</tr>
</tbody>
</table>

a Per liter, 50 mL of sodium dodecyl sulfate, 17 mmol of NaH₂PO₄, and 8 mmol of NaCl, pH 7.

b Blocking solution diluted 10-fold in water.

c Per liter, 100 mmol of Tris, 100 mmol of NaCl, and 10 mmol of MgCl₂, pH 9.5.

d From Millipore Flex™ luminescent kits.

Fig. 1. PCR amplification (A) and direct sequencing (B) of a 198-bp cDNA fragment encompassing the bcr-abl junction in a CML patient RNA was isolated from nucleated cells of peripheral blood. After reverse transcription with primer A, the bcr-abl junction was amplified by PCR with two pairs of nested primers: A, B and C, D (see text). The biotinylated 198-bp fragment (A), after coupling to streptavidin-coated magnetic beads, was subjected to direct sequencing, followed by chemiluminescent detection of the sequencing products. (B) Sequencing pattern located in the abl portion of the DNA fragment amplified (nucleotides 34 to 104 of exon 11 (11): 5'AGGCCTCAGGGTCTGAGTGAAGCcGCTCGTrGGAACTCCAAGGA&AACCTrCTCGC-

ments we observed adsorption of a certain amount of magnetic beads on tube walls after treatment of the bound PCR product with NaOH, 0.2 mol/L. Thereafter we used siliconized Eppendorf tubes, allowing a better recovery of the beads.

The method described does not require cloning the DNA before sequencing. It involves—after in vitro amplification of the DNA fragment with two primers, one of which is 5'-biotinylated—immobilization of the fragment to a solid support, followed by Sanger sequencing with a 5'-biotinylated sequencing primer. The chemiluminescent detection chemistry described above, although comparable in sensitivity (4), has several advantages over existing radioisotopic detection chemistries. Among them is the absence of manipulation of radioisotopes, which is important for the safety of technicians when DNA sequencing becomes a procedure routinely used in clinical laboratories. Biotin-labeled oligonucleotides and sequencing reactions are stable indefinitely if kept in proper conditions, and are not subjected to decay, radiolysis, or light-bleaching. That makes it possible to check a result at any time and increases the diagnostic reliability.

Detection times are short and represent a great improvement over the range of hours needed for detection when radioisotopes are used. Figure 1B shows a sequencing pattern after a 10-min exposure.

The equipment used here is available in any laboratory set up for molecular biology. Transfer of the gel to filter paper (we run 60-cm-long gels) and the membrane-processing steps required for the chemiluminescent detection could be considered the most difficult points of the described method. However, with training, these operations rapidly become easy, and the inconveniences are negligible considering the gain in time and safety. Determining a patient's DNA sequence takes an average of 3.5 days after leukocyte preparation, RNA extraction, cDNA synthesis, and application of the protocol described here.

In summary, this PCR direct solid-phase sequencing of DNA with use of magnetic beads as the solid support and chemiluminescent detection of the sequencing products allows fast, simple, and nonradioactive manual DNA sequencing that is particularly suitable for use in clinical laboratories.

This work was supported by grants from the Faculty of Medicine Paris XI; Assistance Publique–Hôpitaux de Paris, clinical research contract no. 912104; and Association pour la Recherche sur le Cancer (ARC) contract no. 6258. We are grateful to S. Riva for technical assistance. We thank J. L. Misset and his team for clinical collaboration, E. May, P. Paterlini, and C. Brechot for helpful advice and discussion, and E. Yonish Rousch for reading the manuscript.

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When cyanide poisoning is treated with a methemoglobin-forming agent, oxidative metabolism is protected at the expense of the oxygen capacity of the blood. The affinity of methemoglobin for CN\(^{-}\) is high enough to compete with cytochrome oxidase, which protects the latter from becoming blocked, but all hemoglobin used for this purpose is lost for the transport of oxygen. Therefore, the fractions of the various hemoglobin derivatives present in the blood should be carefully monitored during this kind of treatment. After we had developed a multicomponent spectrophotometric method for this purpose, we studied the feasibility of using a modified commercial six-wavelength hemoglobin photometer (Radiometer OSM3) for easy and rapid analysis of methemoglobin and methemoglobin cyanide in small samples of blood. All conditions appeared to be fulfilled for the construction of a practical multicomponent photometer for reliably monitoring methemoglobin therapy in patients with cyanide poisoning, even in the presence of carboxyhemoglobin, as often occurs in fire victims.

Indexing Terms: toxicology • cytochrome oxidase • carboxyhemoglobin • multiple-wavelength analysis • spectrophotometry

Acute cyanide (CN\(^{-}\)) poisoning is often treated by injection of methemoglobin (MetHb)-forming agents (1–

4). Through its high affinity for CN\(^{-}\), MetHb is able to keep CN\(^{-}\) away from the cytochrome \(\varepsilon_3\) component of cytochrome oxidase (EC 1.9.1.3), which otherwise would become blocked. Thus time is gained for detoxication through the conversion of cyanide to thiocyanide (CNS\(^{-}\)), mediated by the mitochondrial enzyme rhodanese (thiosulfate cytochrome sulfurtransferase; EC 2.8.1.1). The enzyme can handle quite large amounts of CN\(^{-}\), but under natural conditions the reaction is limited by a scarcity of substrate. Effective detoxication is therefore achieved only by the additional administration of an adequate amount of thiosulfate as a source of sulfur. The CNS\(^{-}\) formed is relatively nontoxic and easily eliminated by the kidneys.

For successful treatment, a considerable amount of MetHb should be formed. To bind all CN\(^{-}\) from an ingested amount of 1 g of KCN, MetHb must be 30% of the total hemoglobin in a 70-kg man with a total hemoglobin concentration of 150 g/L (5). The protection of cytochrome oxidase thus is achieved at the cost of a considerable decrease in the oxygen capacity of the blood: oxygen transport capability is traded for maintaining oxidative energy release. This inevitably limits the allowable degree of MetHb formation and necessitates careful monitoring of MetHb and methemoglobin cyanide (CNMetHb) from the beginning of the first injection of the MetHb inducer to the end of the therapy.

Nonstandard abbreviations: MCA, multicomponent analysis; O\(_2\)Hb, oxyhemoglobin; HHb, deoxyhemoglobin; COHb, carboxyhemoglobin; MetHb, methemoglobin; CNMetHb, methemoglobin cyanide; and SulHb, sulfhemoglobin.