Table 7. Cuvette-Related Carryover

<table>
<thead>
<tr>
<th>Assay cuvettes</th>
<th>1–21: total bilirubin&lt;sup&gt;a&lt;/sup&gt;</th>
<th>1–21: urea&lt;sup&gt;b&lt;/sup&gt; (U&lt;sub&gt;i&lt;/sub&gt;)</th>
<th>22–42: urea&lt;sup&gt;b&lt;/sup&gt; (U&lt;sub&gt;j&lt;/sub&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean, mmol/L</td>
<td>7.52</td>
<td>6.86</td>
<td>6.74</td>
</tr>
<tr>
<td>SD, mmol/L</td>
<td>0.16</td>
<td>0.11</td>
<td>0.11</td>
</tr>
<tr>
<td>CV, %</td>
<td>2.33</td>
<td>1.63</td>
<td>1.63</td>
</tr>
<tr>
<td>Bias, %</td>
<td>1.78</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

<sup>a</sup> Modified Jendrassik–Grof method; urea (additive) present in the reaction mixture at ~2.6 mmol/L.

<sup>b</sup> Determined with Axon reagents.

<sup>c</sup> [(U<sub>i</sub> - U<sub>j</sub>)/U<sub>j</sub>] × 100.

SD of 5.57–5.72 mmol/L, for a negative carryover effect of 1.9%. Given the very low within-run SDs for these assays, we consider this type of carryover also to be insignificant. The negligible bias value reported in Table 7 for cuvette carryover documents that, even in the extreme assay conditions we used, there was no cuvette-related carryover. This proves the high efficiency of the cuvette-washing procedure of the Axon system.

**Recovery in control sera.** The mean values measured in the control sera were between 95% and 105% of the manufacturers’ assigned values.

**Discussion**

During the 5-month evaluation period, we collected ~10 000 data points, encountering only one problem: the instrument stopped because of a malfunction of the level-sensor of the reagent 1 probe, but this was solved within 24 h by the manufacturer’s service personnel. The operation proved to be simple, and maintenance was not time consuming. The results obtained proved the overall analytical reliability of the system, which is compact and easy to use on a 24-h basis. The instrument software is quite flexible, and the management of stored data allows valuable chances of control to the operator. The settings for the built-in methods are easily understandable; user-defined methods may also be applied easily. Moreover, the reagent configuration can be quickly modified, when desired, because of the software operations of loading/unloading methods.

Thus, because of its reliability and practicability, we conclude that the Axon system is well suited for laboratories with various needs.

We thank Laura Porati for the dedicated completion of the experimental work. We also greatly appreciate the support of G. Tarenghi and A. Fumagalli (Scientific Dept., Bayer Diagnostic SpA, Cavenago Brianza) for the statistical evaluation.

**References**


**Two Novel Nonradioactive Polymerase Chain Reaction-Based Assays of Dried Blood Spots, Genomic DNA, or Whole Cells for Fast, Reliable Detection of Z and S Mutations in the α1-Antitrypsin Gene**

Brage Storstein Andresen, Inga Knudsen, Peter K. A. Jensen, Kirsten Rasmussen, and Niels Gregersen

Two new nonradioactive polymerase chain reaction (PCR)-based assays for the Z and S mutations in the α1-antitrypsin gene are presented. The assays take advantage of PCR-mediated mutagenesis, creating new diagnostic restriction enzyme sites for unambiguous discrimination between test samples from individuals who are normal, heterozygous, or homozygous for the mutations. We show that the two assays can be performed with purified genomic DNA as well as with boiled blood spots. The new assays were validated by parallel testing with a technique in which PCR is combined with allele-specific oligonucleotide (ASO) probes. In all cases tested the results obtained by the different techniques were in accordance. The new assays can be used for prenatal diagnostics and can be performed directly with boiled tissue samples. Because the new assays are easy to perform and reliable, we conclude that they are well suited for routine diagnosis.

**Additional Keyphrases:** fetal status • heritable disorders • allele-specific oligonucleotide probes compared

α1-Antitrypsin (α1-AT) is a 52-kDa glycoprotein produced mainly in liver tissue. It serves as the major

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2 Institute of Human Genetics, University of Aarhus, DK-8000 Aarhus C, Denmark.

Received January 24, 1992; accepted May 1, 1992.

2 Nonstandard abbreviations: α1-AT, α1-antitrypsin; PCR, polymerase chain reaction; ASO, allele-specific oligonucleotide; and ARMS, Amplification Refractory Mutation System.
inhibitor of neutrophile elastase, a powerful proteolytic enzyme stored in neutrophile leucocytes (1). α1-AT deficiency (reviewed in 2) is an autosomal recessive inherited disease, present in 1 in 1000 newborns in Northern Europe. Individuals with α1-AT deficiency are at risk of developing early-onset emphysema, because lack of the inhibitor leaves the structural framework of the lungs unprotected (3). In addition, patients homozygous for the Z mutation of the gene have an increased risk of developing liver disease in childhood, which may progress to cirrhosis and early death. Diagnosis of α1-AT deficiency is therefore important for estimating the risk for disease and for making recommendations for augmentation therapy. Furthermore, prenatal diagnosis of the Z mutation can be justified in certain families. In families with α1-AT deficiency in which serious cases of liver disease have been observed previously, the risk of a subsequent homozygous child developing liver disease is ~40% (4).

α1-AT deficiency results from a variety of mutations in the α1-AT gene. At least 17 different disease-associated mutations in this gene are currently known (2). In the vast majority of cases, the disease is caused by homozygosity for the Z mutation or by compound heterozygosity (Z mutation in one allele and S mutation in the other). The Z mutation (E342K) is a G9999 to A transition in exon V of the α1-AT gene; the S mutation (E264V) results from an A7877 to T transversion in exon III (5, 6).

The various α1-AT genotypes have been identified by restriction fragment length polymorphism analysis (4, 7) of purified genomic DNA and by allele-specific oligonucleotide (ASO) probes (8). Since the development of the polymerase chain reaction (PCR), several PCR-based assays have been constructed, including PCR combined with ASO probes (9, 10), direct sequencing of PCR-amplified DNA fragments (11), the Amplification Refractory Mutation System (ARMS) (12), and PCR combined with cleavage by RNase A (13). All of these assays have shortcomings for use in routine diagnosis. They are too laborious, involve the use of radioactivity, or are unreliable. To overcome these shortcomings, we have designed two new nonradioactive PCR-based assays to detect Z and S mutations in the α1-AT gene.

The new assays are based on the principle of using PCR-mediated mutagenesis (14) to create new restriction enzyme sites; this enables discrimination between normal and mutation-bearing alleles by restriction enzyme digestion of PCR-amplified fragments. We previously used this technique to detect the most frequent mutation in the medium-chain acyl-CoA dehydrogenase gene (15, 16) and to detect the apoB100 3500 mutation in the gene for apolipoprotein B100 (17).

Here we compare the new assays with the conventional ASO-probe assays for the Z and S mutations by typing purified genomic DNA from patients with diagnosed α1-AT deficiency, from patients with pulmonary emphysema, and from a family whose members exhibit both the Z and S mutations. We also show that the new assays can be performed directly with boiled blood spots, making diagnosis fast and easy. Finally, we report use of the new assays for prenatal diagnosis of two cases of α1-AT deficiency in the same family, and show that the new assays can also be performed directly with boiled samples of chorionic villus tissue.

Materials and Methods

Materials

Primers for the PCR amplifications and oligonucleotide probes were synthesized with a DNA Synthesizer from Applied Biosystems Inc. (Foster City, CA). For the PCR amplifications we used an Automated Thermal Cycler from Perkin-Elmer Cetus (Norwalk, CT). Mononucleotides dATP, dCTP, dGTP, and dTTP for the PCR amplification were from Sigma Chemical Co. (St. Louis, MO). Recombinant Taq polymerase was from Perkin-Elmer Cetus. Restriction enzymes Asp700 and TaqI were purchased from Boehringer, Mannheim, FRG; XmnI was from Stratagene, La Jolla, CA. α1-Casein was obtained from Merck, Darmstadt, FRG. Acrylamides were from Serva Feinbiochemica, Heidelberg, FRG. The size marker was αC, 857 DNA digested with DraI.

Sources of DNA. Blood samples from patients with pulmonary emphysema, patients with α1-AT deficiency, and family members of family I and family II were collected by Kirsten Bruun Petersen, Gert Bruun Petersen, and Ronald Dahl. The typing of the emphysema patients and of family II was published previously (18). Material for the two cases of prenatal diagnosis in family III was collected by Peter Skovbo, Department of Gynecology, Ålborg Sygehus, Ålborg, Denmark.

Procedures

Preparation of DNA. Genomic DNA was isolated by standard methods (19) from blood samples, placental tissue, and chorionic villus biopsies, and from cultured cells from the chorionic villus biopsies as well as from cultured cells from the aborted fetuses. Blood spots were prepared from either fresh or frozen blood (frozen from 1 week to 8 years) by applying ~50 μL to thick Whatman filter paper. Filter paper pieces (2.5 × 2.5 mm) corresponding to 2-5 μL of blood were first fixed with methanol; the DNA was then liberated by boiling each piece in 50 μL of sterile water for 15 min (16). Either tissue samples (~2 mm³) from a chorionic villus biopsy or from cultured cells from chorionic tissue were washed twice in a solution of, per liter, 0.1 mol of NaCl, 0.01 mol of Tris·HCl, and 1 mmol of EDTA, pH 8.0. The samples were then boiled for 2 min in 50 μL of a solution of, per liter, 0.1 mol of NaOH, 2 mol of NaCl, and 20 mL of Triton X-100. After centrifugation we used 1-2.5 μL of the supernate for PCR amplification.

PCR amplifications. All PCR amplifications were performed with standard buffer (10× amplification buffer: 0.5 mol of KCl, 0.1 mol of Tris·HCl, 15 mmol of MgCl₂, and 0.1 g of gelatin per liter, pH 8.3) in a 100-μL total volume containing DNA template, oligonucleotide primers, dNTPs (20.0 nmol of dATP, dCTP, dGTP, and dTTP), and 2 U of Taq polymerase. Before amplification, the PCR mixtures were overlaid with 75 μL of paraffin
oil. The details of the various PCR amplifications are described in Table 1.

A blank amplification containing all reagents but no sample DNA was always included in the experiments to check for the presence of contaminating DNA from cloned or previously amplified DNA.

**PCR combined with ASO probe analysis for the Z and S mutations.** A 148-bp fragment containing the region harboring the site for the S mutation and a 139-bp fragment harboring the Z mutation site were co-amplified in one PCR procedure with use of the four oligonucleotide primers p1, p2, p3, and p4 (Table 2). After the amplification, 1-μL samples of the PCR mixture, containing the two amplified fragments, were denatured and applied at corresponding positions on four Zeta nylon membranes. The membranes were hybridized separately with each of four different [γ-32P]ATP end-labeled ASO probes. Two probes were used for each of the two mutations: one probe with the normal sequence (S<sub>M</sub>-probe or Z<sub>M</sub>-probe) and one probe with the mutant sequence (S<sub>p</sub>-probe or Z<sub>p</sub>-probe) (Table 2). After hybridization, the membranes were washed separately at low stringency, followed by stringent washing at temperatures specific for each probe. The bound probes were made visible by autoradiography. Details of the ASO probe analysis for the S and Z mutations in PCR-amplified DNA were described previously (9, 18).

**PCR amplification mutagenesis and diagnostic restriction enzyme digestion for the Z and S mutations.** The principles of the new assays are described in Figure 1. For convenience we optimized both assays so that the amplifications could be performed with the same PCR program. Thus, the two assays can be performed simultaneously, making diagnosis for the two mutations easier.

In the S mutation assay, the p7702 primer (antisense) contains an AA instead of a TT dinucleotide at position 7683–7684. This change results in the creation of a *XmaI* site (GAANNNTTC) when the normal sequence is copied by PCR, because a T is inserted at position 7677. Alleles with the S mutation instead have an A at

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### Table 1. PCR Conditions

<table>
<thead>
<tr>
<th>ASO probe</th>
<th>PCR mutagenesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primers</td>
<td>p7553+p7702</td>
</tr>
<tr>
<td>Template</td>
<td>p9966+p10063</td>
</tr>
<tr>
<td>Primer amount (each)</td>
<td>60 pmol</td>
</tr>
<tr>
<td>Cycling conditions</td>
<td>1 cycle of:</td>
</tr>
<tr>
<td></td>
<td>95 °C, 8 min;</td>
</tr>
<tr>
<td></td>
<td>55 °C, 5 min;</td>
</tr>
<tr>
<td></td>
<td>72 °C, 5 min;</td>
</tr>
<tr>
<td></td>
<td>29 cycles of:</td>
</tr>
<tr>
<td></td>
<td>95 °C, 1 min;</td>
</tr>
<tr>
<td></td>
<td>55 °C, 2 min;</td>
</tr>
<tr>
<td></td>
<td>72 °C, 5 min;</td>
</tr>
<tr>
<td></td>
<td>1 cycle of:</td>
</tr>
<tr>
<td></td>
<td>72 °C, 9 min</td>
</tr>
</tbody>
</table>

### Table 2. Oligonucleotides in the Assays

**S mutation primers**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>p1</td>
<td>5'-CAATGCCACCGCCATCCTTCTTCCGTGCTG-3'</td>
</tr>
<tr>
<td>p2</td>
<td>5'-TGTGGGCAGCTTTCTGGTCACCTCAGGT-3'</td>
</tr>
<tr>
<td>p7553</td>
<td>5'-GATGATATCGTGATGGAGAACA1Tr-3'</td>
</tr>
<tr>
<td>p7702</td>
<td>5'-GATGATACGTGGGTGAGAACA1Tr-3'</td>
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</tbody>
</table>

**Z mutation primers**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>p3</td>
<td>5'-CCTGGGATCAACCCATACCGTCTGCTGCTG-3'</td>
</tr>
<tr>
<td>p4</td>
<td>5'-CGGAGGTGTCATGGAAGCGTGGTCTGCTG-3'</td>
</tr>
<tr>
<td>p9966</td>
<td>5'-ATAGGGTGTCGACTGATCGTCC-3'</td>
</tr>
<tr>
<td>p10063</td>
<td>5'-GACCTTGAACCTCGAGGGGTGACG-3'</td>
</tr>
</tbody>
</table>

**ASO probes for the S mutation site**

<table>
<thead>
<tr>
<th>Probe</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>S&lt;sub&gt;M&lt;/sub&gt;</td>
<td>5'-CAGCACCTGAGAATGAACTC-3'</td>
</tr>
<tr>
<td>S&lt;sub&gt;B&lt;/sub&gt;</td>
<td>5'-CAGCACCTGAGAATGAACTC-3'</td>
</tr>
</tbody>
</table>

**ASO probes for the Z mutation site**

<table>
<thead>
<tr>
<th>Probe</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z&lt;sub&gt;M&lt;/sub&gt;</td>
<td>5'-ACCACCGAGAAGGGACT-3'</td>
</tr>
<tr>
<td>Z&lt;sub&gt;Z&lt;/sub&gt;</td>
<td>5'-ACCACCGAGAAGGGACT-3'</td>
</tr>
</tbody>
</table>

Nucleotides corresponding to the mutation site are underlined.
Fig. 1. Principle of the assay for the S mutation (upper panel) and for the Z mutation (lower panel)

Upper panel: In the S mutation assay, PCR amplification with the two primers p7553 and p7702 produces a 149-bp fragment of exon III of the α₂-AT gene. The p7702 primer introduces a diagnostic XmnI site (GAANNNNNTG) only in the PCR product from normal alleles but not in that from alleles bearing the S mutation. The presence or absence of this XmnI site makes it possible to distinguish between PCR-amplified normal and S mutation-bearing alleles after cleavage with XmnI followed by polyacrylamide gel electrophoresis. The p7553 primer introduces a XmnI site in all amplification products; therefore, this site serves as an internal control for the restriction enzyme cleavage. After cleavage with XmnI, amplified S mutation-bearing alleles are 133 bp and amplified normal alleles are 111 bp.

Lower panel: In the Z mutation assay, PCR amplification with the two primers p9966 and p10063 produces a 97-bp fragment of exon V of the α₂-AT gene. The p9966 primer introduces a TaqI site (TCGA) only in the PCR product from normal alleles but not in that from alleles bearing the Z mutation. The presence or absence of this TaqI site makes it possible to distinguish between normal and Z mutation-bearing alleles after cleavage with TaqI followed by polyacrylamide gel electrophoresis. The p10063 primer introduces a TaqI site in all PCR products; therefore, this TaqI site serves as an internal control for restriction enzyme cleavage. After cleavage with TaqI, amplified Z mutation-bearing alleles are 86 bp and amplified normal alleles are 64 bp.

The restriction enzyme sites are not created when they are copied. The nucleotides TT at positions 7565 and 7566 are changed to AA, and A at position 7571 is changed to T by the p7553 primer (sense), thus creating an XmnI site in both amplified normal and mutation-bearing alleles. This site serves as an internal control of restriction enzyme digestion.

In the Z mutation assay, the p9966 primer (sense) contains a T instead of an A at position 9986. This change creates a TaqI site (TCGA) when the normal sequence with a G at position 9989 is copied, but not when the Z mutation sequence with an A at position 9989 is copied. By changing the G at position 10050 to an A, the p10063 primer (antisense) creates a TaqI site, which serves as an internal control of cleavage efficiency in all alleles copied. The primer sequences, with the mismatching nucleotides underlined, are shown in Table 2.

Before the restriction enzyme digestion, we tested the performance of the PCR procedure, including the "blank" amplification, by subjecting samples of the PCR products to electrophoresis in a 3% agarose gel.

Restriction enzyme digestion. Samples of 7–20 μL were digested overnight in a 50-μL total volume containing 5 μL of α₁-casein (1 g/L) and 10 U of the respective restriction enzyme. After 14–16 h, we added
10 U more of enzyme and continued the digestions for 1–2 h. For digestion with XmnI, we used the buffer recommended by the supplier. For digestion with TaqI, we found the best results with 1× low-salt buffer at 65 °C (10× low-salt buffer: 0.1 mol of MgCl₂, 0.01 mol of dithiothreitol, and 0.1 mol of Tris·HCl per liter, pH 7.5). Afterwards, we electrophoresed the digested samples and undigested samples in 16% polyacrylamide gels, staining the bands with ethidium bromide.

Results

Using various sources of DNA, we found that the two new assays functioned well and were reliable. To compare the performance of the new assays with the conventional PCR/ASO probe assays, we used both methods to test purified genomic DNA from family I. The results of the typing for the S mutation (Figure 2) and the Z mutation (Figure 3) with the new assays (A panels) and the PCR/ASO probe assays (B panels) were identical in all family members. With the S mutation assay, homozygous persons showed only one band of 133 bp (Figure 2A, lane C3), persons who did not have the S mutation showed only one band of 111 bp (Figure 2A, lane C1), and persons heterozygous for the S mutation showed both the 133-bp band and the 111-bp band (Figure 2A, lane C2). In the Z mutation assay, a single band of 86 bp was seen when the person was homozygous for the Z mutation (Figure 3A, lane C4), a single band of 64 bp was seen when the person did not have the Z mutation (Figure 3A, lane C1), and persons heterozygous for the Z mutation showed both the 86-bp band and the 64-bp band (Figure 3A, lane C2). This clearly illustrates that individuals who are heterozygous for the respective mutations can be distinguished unambiguously from normal persons and from subjects who are homozygous for the mutation in question. Note that alleles with the S mutation will appear “normal” in the Z mutation assay, and vice versa.

During optimization of the S mutation assay, we also tested the performance of the XmnI isoschizomeric enzyme, Asp700. Despite testing several different buffers and various amounts of restriction enzyme, we found that optimal cleavage efficiency could be achieved only with the XmnI enzyme. Thus, in cases where different isoschizomeric restriction enzymes can be used, it might be advantageous to test the performance of all alternative enzymes.

To be able to diagnose the two mutations more quickly and easily, we optimized the PCR conditions so that the new assays could be performed directly with DNA liberated from boiled blood spots (Guthrie cards). Because the amounts of DNA liberated from blood spots are generally very low and variable, we needed to make the PCR amplification more specific (Table 1). To optimize the new assays, we used blood spots obtained from family members from family II, a family previously examined by isoelectric focusing and by the PCR/ASO probe assay (18). A comparison of the genotypes based
The results were compared with results obtained previously (18, and unpublished results), when genomic DNA samples from the same patients were tested with the traditional PCR/ASO probe assays. In all cases, the results obtained with the two new assays were in complete accordance with the results obtained previously. The age of the blood spots or of the frozen blood did not seem to influence the performance of the two assays.

After having confirmed the good performances of the new assays, we used them for prenatal diagnoses. Family III had previously had a seriously affected child, who is homozygous for the Z mutation. By testing purified genomic DNA from the parents and the index patient, we confirmed the previous diagnosis (obtained by the PCR/ASO probe assay), showing that the parents are heterozygous carriers (Figure 5I, lanes 1 and 2) and that the index patient is homozygous for the Z mutation (Figure 5I, lane 3). The first prenatal diagnosis was performed with purified genomic DNA from chorionic villus biopsies obtained from each of two dizygotic twin fetuses. In the Z mutation assay, the DNA from both fetuses displayed a single band at 86 bp (Figure 5I, lanes 4 and 5), showing that both samples were homozygous for the Z mutation. On this basis, the parents chose to abort the twin fetuses. After termination of the pregnancy, the diagnosis was verified in DNA samples from cultured cells of the aborted fetuses (Figure 5I, lanes F1 and F2) and in DNA prepared from a sample of placental tissue obtained after the abortion (Figure 5I, lane P).

The second prenatal diagnosis was performed 4 months later in a new pregnancy of the mother in the same family (family III). This time we performed the diagnosis with DNA isolated from a chorionic villus biopsy of the fetus (Figure 5II, lane CVA) and directly with a boiled sample of the same chorionic villus biopsy (Figure 5II, lane CVB). We also assayed DNA isolated from cultured cells from the chorionic villus biopsy (Figure 5II, lane CVc) and directly boiled samples of the cultured cells (Figure 5II, lane CVd). Because only the 64-bp band was observed in all the fetal samples, we concluded that this fetus was homozygous for the normal allele and the pregnancy was continued.

Fig. 4. S mutation assay (upper electropherogram) and Z mutation assay (lower electropherogram) performed on blood spots from family II on the results obtained by the PCR/ASO probe assays of purified genomic DNA (summarized in Figure 4) with the results obtained by the new methods for assays of blood spots (Figure 4) shows that the diagnoses totally agree. Moreover, the results obtained when the two new assays are performed with blood spots (Figure 4) are as easy to interpret as the results obtained by the new assays performed with purified genomic DNA from members of family I (Figures 2 and 3).

Purified genomic DNA samples (in some cases also blood spots) from 16 patients with a clinical history of pulmonary emphysema and from 8 patients with diagnosed α1-AT deficiency were tested with the new assays.

Fig. 5. Prenatal diagnosis in family III performed with the new assay for the Z mutation Ethidium bromide-stained polyacrylamide gel after electrophoresis of TaqI-digested and undigested PCR products. (I) First prenatal diagnosis: C1, control MM; C2, control SS; C3, control ZZ; B, blank amplification. Lane 1, mother; 2, father; 3, index patient; 4, 5, chorionic villus DNA from the twin fetuses; F1, F2, DNA from cultured cells of the aborted twin fetuses; P, placental DNA from the aborted twin fetuses. (II) Second prenatal diagnosis: UC, uncleaved PCR product; C4, control MM; C5, control ZZ; C6, control SS; C7, control ZZ. Lane 1, father; 2, mother; CVa, chorionic villus DNA from the fetus; CVb, whole cells from the chorionic villus biopsy; CVc, DNA from the cultured cells from the chorionic villus biopsy; CVd, cultured cells from the chorionic villus biopsy; B, blank amplification.
The S mutation assay showed only a single band of 111 bp in all samples tested (results not shown). Therefore, we concluded that all samples were normal with respect to this site.

Discussion

Diagnosis of $\alpha_1$-AT deficiency has traditionally been based on isoelectric focusing and measurement of the $\alpha_1$-AT protein concentration in serum. This method is fast and simple, but interpretation of the banding pattern obtained by isoelectric focusing is difficult and requires specially trained personnel. Alternative methods of diagnosis involve determination of the disease-causing mutations in DNA samples by PCR-based assays (9–13). The most widely used techniques for this are PCR combined with ASO probes and the ARMS assay.

PCR combined with ASO probes for the Z and S mutations (9) is very reliable, but is labor intensive; for routine use, it requires the use of two radioactively labeled probes. Nonradioactive (biotin-labeled) probes have also been used (10), but this system is not sufficiently robust for routine use. The ARMS assay (12) may seem like a good alternative to the PCR/ASO probe assays: it is nonradioactive and is more easily performed. Interpretation of the results obtained with the ARMS technique is based on distinguishing between successful amplification and failure to amplify in pairs of reactions.

We previously tried using the ARMS technique to detect the S and Z mutations, as described by Newton et al. (12), but in routine use the diagnosis was not always reliable, even though an internal control for amplification was included. It became clear that the presence of a band was often determined by the initial amount of DNA template present and by how many cycles of PCR were performed. Consequently, the ARMS method does not seem suited for use with blood spots as the source of DNA, because the amounts of DNA released from blood spots are generally low and variable.

As a compromise between ease of performance and reliability, we had until recently considered PCR combined with radioactively labeled ASO probes as the method of choice for routine diagnosis of the Z and S mutations (18, 20). However, radiolabeled ASO probes are expensive and the procedure is laborious. The new assays presented here alleviate these drawbacks. Moreover, in the families studied, the results obtained with the new assays agreed with the PCR/ASO probe assays, and identical segregation was found. Total agreement between results obtained with the new assays and with the PCR/ASO probe assays was also found for tests involving an additional 24 patients. We therefore conclude that the two new assays presented here are just as reliable as the PCR/ASO probe assays, and may therefore be used for routine diagnosis.

We have also shown that the new assays can be used for carrier detection and prenatal diagnosis in an affected family (family III). The results obtained for family III illustrate that prenatal diagnosis for $\alpha_1$-AT deficiency can be performed with DNA from chorionic villus biopsies. Moreover, the fact that one can perform the two new assays directly on chorionic villus samples means that the assays can be performed very quickly, because the DNA need not be purified before analysis. Furthermore, the new assays, in contrast to the ARMS method, can be performed with boiled blood spots to give unambiguous results. This advantage makes the new assays very rapid indeed, because no purification of genomic DNA is required. The use of blood spots is also desirable because they are easier to ship and store than are blood samples.

In our experience, the most critical step in the new assays is the restriction enzyme digestion step. Therefore, when constructing the assay, great care should be taken to ensure that the preconditions for complete digestion are as good as possible—avoiding production of primer–dimer product in the PCR, and choosing the optimal restriction enzyme site to create without limiting whether the restriction enzyme site should be created in the mutant sequence or in the normal sequence (21).

Another very important element that is often ignored (22–25) is inclusion of control sites for restriction enzyme cleavage. Including a control site will indicate when digestion is incomplete, thereby avoiding problems with false-negative or false-positive results. The control sites in our assays are placed in such a way that the cleaved-off fragments are a different size (16 bp in the S assay and 11 bp in the Z assay) from the fragment cleaved off at the mutation site (22 bp in both the S and Z assays). Given this difference in fragment sizes, we can distinguish unambiguously between cases of incomplete digestion and true heterozygosity. In contrast to the problems we encountered in optimizing the restriction enzyme digestion step, the PCR amplification step was less difficult to optimize. Even when one of the primers contained a mismatch as close as at position 2 from the 3′-terminus (p9666 primer in the Z mutation assay) or had as many as three mismatching nucleotides located in the middle (p7553 primer in the S mutation assay), successful amplification was easy to attain.

Because PCR can tolerate rather drastic mismatching, we believe it would be easy to manipulate the sequences around any given mutation site by PCR-based mutagenesis in such a way that the sequences can be recognized and discriminated by restriction enzymes. This belief has thus far been corroborated by the fact that the method has been used to detect mutations in the genes for $\beta$-globin (21), cystic fibrosis (22), cystic fibrosis and rhodopsin (retinitis pigmentosa) (25), apolipoprotein B100 (17, 23, 24), and medium-chain acyl-CoA dehydrogenase (15, 16). Therefore, we postulate that this method can be universally applied for detection of point mutations and small insertions or deletions.

This work was supported by the Danish Medical Research Council and the Danish Center for Human Genome Research. We thank Lars Bolund for helpful discussion of the manuscript.
References


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Standardization with Synthetic 22-kDa Monomer Human Growth Hormone Reduces Discrepancies between Two Monoclonal Immunoradiometric Assay Kits

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Discrepancies among different methods for assaying human growth hormone have been described in various studies. The two major sources of discordant results are the heterogeneity of the antibodies and the different standardization bases used by the assay manufacturers. We propose standardizing assays with 22-kDa biosynthetic monomer human growth hormone diluted with the diluents supplied by the kit manufacturers. In a study of two monoclonal immunoradiometric assays (Hybritech, specific for the 22-kDa monomer; Sorin, recognizing also a 20-kDa variant hormone), standardization with 22-kDa monomer human growth hormone reduced by 63% the differences in results for 44 serum samples from children. The use of 22-kDa human growth hormone as a common standard, highly pure and easily available in large quantities, could help limit the interpretative problems in growth diagnostics.

Additional Keyphrases: variation, source of · somatotropin

Disagreement and discrepancies among different methods for assaying human growth hormone (hGH, somatotropin) have been reported by various research-