

Rapid PCR Real-Time Genotyping of M-Malton α_1 -Antitrypsin Deficiency Alleles by Molecular Beacons

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Abstract: α_1 -Antitrypsin deficiency is an autosomal codominant inherited disorder, with increased risk of developing lung and liver disease. The large majority of subjects affected by α_1 -antitrypsin deficiency carry the PIZZ or PISZ genotypes, which can be easily detected using several molecular methods. Another pathologic allele, the M-Malton variant (also known as Mnichinan and Mcagliari), can mimic the Pi Z clinical phenotype, but this α_1 -antitrypsin deficiency variant is not easily recognizable and, therefore, seems to be more underrecognized than the Z or S alleles. We report the development of a rapid qualitative fluorescent real-time PCR assay designed for the detection of the M-Malton α_1 -antitrypsin deficiency alleles using 2 specific molecular beacons. The assay is able to detect in a single tube the homozygous as well the heterozygous genotypes. The procedure combines the great sensitivity of the polymerase chain reaction, the specificity provided by allele-specific molecular beacons, and the throughput of a multicolour fluorescence detection procedure. This technique will be useful for research and molecular diagnostic laboratories involved in the study of α_1 -antitrypsin deficiency-related diseases.

Key Words: α_1 -antitrypsin deficiency, M-Malton variant, real-time PCR, molecular beacon

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α_1 -Antitrypsin deficiency (AATD) is an autosomal hereditary disorder caused by several mutations in the AAT gene. Its major clinical manifestations include pulmonary emphysema in adults and liver disorders in children as well as in adults.¹ Based on known gene frequency data, AATD seems to be one of the commonest inherited disorder worldwide. In the Caucasian population, but also among other ethnic groups, the large majority of subjects affected by AATD carry the PIZZ or PISZ genotypes,^{2,3} which can be easily diagnosed at protein level (isoelectrofocusing)⁴

or using PCR-restriction fragment length polymorphism⁵ or with a more sensitive and simple real-time fluorescence PCR method.^{6,7}

For this reason, the majority of the studies on the molecular and clinical pathologic pictures associated with AATD have been made in subjects carrying the PIZZ or PISZ phenotype. Nevertheless, there are several AAT alleles, other than the PIZ and the PIS, associated with significantly reduced plasma AAT levels.⁸ Such variants are rare, often described as single case reports in the recent literature,^{9,10} and little is known about their epidemiology and even less about the associated clinical phenotypes. Among these rare AATD alleles, a key role is played by the M-Malton (M-Cagliari) variant.¹¹ The mutation of this pathologic variant is characterized by a normal isoelectrophoretic pattern (M-Like variant) and by an entire TTC deletion of 1 of the 2 consecutive 51/52 Phe codons,¹¹ located in a particular hot spot region (M-like region).⁹

From a clinical point of view, the Pi M-Malton mimic the Pi Z clinical phenotype, and, even if it is considered as a rare variant, this “non Z, non S” allele may vary from 10% to 1.7% of all AATD cases founded in 3 different screening studies^{12–14} and it is also considered the most common cause of severe AATD in Sardinia.^{12,15}

There are several molecular methods that can be currently used for the molecular identification of the M-Malton allele like Southern blot hybridization,¹⁶ denaturing gradient gel electrophoresis,¹⁷ DNA haplotypes,¹⁸ and direct sequencing of specific PCR fragments.¹⁹ All these methods are time consuming and/or required facilities or multiple manual steps; for these reasons, the M-Malton variant is not easily recognizable and seems to be more underestimated than the Z or S alleles. Therefore a more suitable, rapid, and sensible method is necessary for a complete knowledge of the real incidence of this variant worldwide. The laboratory protocol described in this paper use a particular stem-loop probe named molecular beacon. These circular oligonucleotides are characterized for a high specificity and a good selectivity when discrimination between different allelic profile in 1 multiplex PCR is necessary.²⁰ Molecular beacons are increasingly being used in many applications involving nucleic acid detection and quantification. In fact, the stem-loop structure of molecular beacons provides a competing reaction for probe-target hybridization that serves to increase probe sensitivity and specificity.²¹ We have developed a fast and specific real-time PCR assay, performed with 2 different molecular beacons, able to detect in

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a single tube the wild type as well as the homozygous or the heterozygous M-Malton ATD genotypes.

MATERIAL AND METHODS

Clinical Samples

All clinical cases of AATD were obtained from the "Sardinian Centre for the study of α_1 -Antitrypsin deficiency," University of Cagliari. Ten homozygous and 10 heterozygous M-Malton and 10 normal samples were selected, and informed consent was obtained from these patients for the genetic analysis. For each sample, AAT serum levels were determined using radial immunodiffusion plates (Dade, Behring, Marburg, Germany). The AAT phenotype was determined by isoelectric focusing (IEF) using a fast system automated electrophoresis apparatus, precasting phastgel dry IEF with pharmlalyte pH 4.2–4.9, and the phastgel protein silver staining kit (Pharmacia Biotech, Uppsala, Sweden).

Synthetic Oligonucleotide Targets

Two complementary single-stranded synthetic oligonucleotides, wild-type and mutated targets, were used for Molecular beacon melting curve analysis (Fig. 1). Oligonucleotides were custom synthesized by MWG Biotech (Ebersberg, Germany, <http://www.mwg-biotech.com/html/all/index.php>).

DNA Extraction

Genomic DNA was extracted from 3 mL of EDTA-anticoagulated blood using standard phenol-chloroforms methods²² or from 3 μ L of EDTA-anticoagulated blood using an ion-chelating resin (Chelex-100, biorad ecc) as previously described.²³

AATD SNPs Detection by Conventional Sequencing

For the specificity control, all samples analyzed with a novel real-time PCR method have been sequenced by a conventional gel-sequencing standard procedure. Exon II^o, III^o, and V^o were PCR-amplified using 3 different sets of primers, as previously described.¹⁹ Direct PCR-DNA sequencing was performed using internal sense and antisense primers

with an automated Alpha Express DNA sequencer (Pharmacia, Uppsala, Sweden), using the termosequenase Fluorescent Labeled Primer Cycle Sequencing Kit (Amersham Life Science).⁹

Primers and Molecular Beacon Design

A schematic representation of the different oligonucleotide primers, molecular beacons, and target sequence used for genotyping the M-Malton allele is shown in Figure 1. The primers were designed using the sequence of the M-like region contained in the second exon of the Human α_1 -antitrypsin gene (GenBanK accession n.K02212).

The theoretical melting temperatures of primers and probe (T_m s), possible oligonucleotide dimmers formation, and/or self-complementarity were calculated using Oligo program version 4 (MedProbe, Oslo, Norway). PCR primers were designed to flank a region of 79 bp of the gene (positions 7494 to 7572).

Molecular beacons were used to detect the real-time PCR amplicon alleles; the arm sequences were designed to form a stable stem hybrid at the annealing temperature of the PCR by *mfold* program (<http://www.bioinfo.rpi.edu/applications/mfold/old/dna/>).^{24,25} T_m folding conditions were as follows: Na^+ 1 mol/L and Mg^{2+} 2.75 mol/L at hybridization temperature of 49°C.

The probes sequences were designed to be complementary to a region within their target region. Fluoresceine (FAM) was used to label the molecular beacon 1 (for wild-type allele). ROX fluorochrome was used to label molecular beacon 2 (for mutated allele). Black hole 1 (BHQ1) and Black hole 2 (BHQ2) were used as quenchers, respectively.

Molecular Beacons Melting Curve Analysis

To determine the capacity for each molecular beacon to detect only a particular allelic sequence, thermal denaturation profiles were studied; these melting curves were also reliable for ensure the correct real-time PCR condition such maximum specificity of annealing temperature (T_a). The reaction was performed with a puReTaq Ready-To-Go PCR Beads (Amersham Biosciences, Buckinghamshire, UK) using the MX 3000 Real time PCR system (Stratagene, www.stratagene.com). The

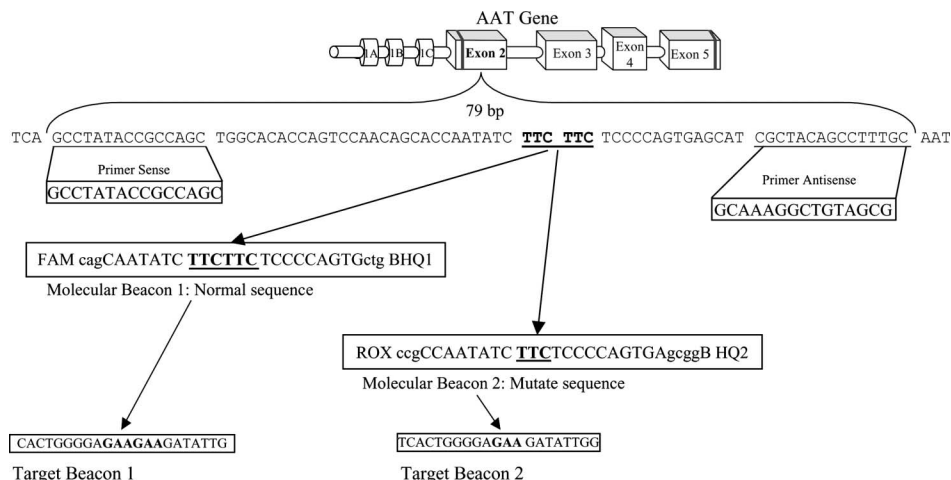


FIGURE 1. Representation of A1AT gene and primers, molecular beacons, and synthetic targets sequences employed in this study.

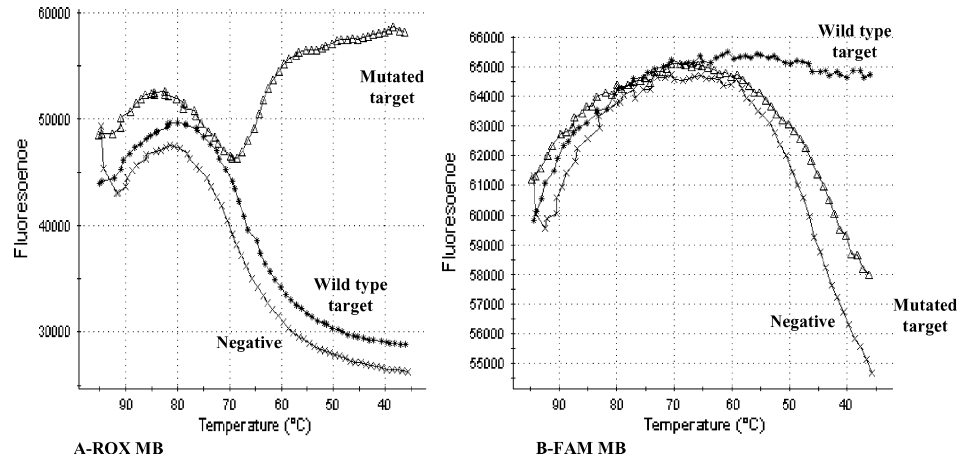


FIGURE 2. Melting curve analysis: (A) ROX mutated molecular beacon and (B) FAM wild-type molecular beacon.

changes in fluorescence of 25 μ L solution, containing 10 μ mol/L of one of the beacon probe with or without 10 μ mol/L complementary single-stranded synthetic oligonucleotides targets, were measured. Molecular beacons temperature denaturation profiles were performed in according to the manufacturer's instructions of Mx 3000 instrument manual (www.stratagene.com). Fluorescent measurements were recorder in continuous with 2 different filter set, excitation/emission: FAM 492/516 and ROX 595/610.

Real-Time PCR Conditions

The real time RT-PCR was performed in puReTaq Ready-To Go PCR Beads (Amersham Biosciences) using the MX 3000 Real time PCR system, according to the manufacturer's instructions. The reaction was conducted in a final volume of 25 μ L containing 2.75 mmol/L MgCl₂, 2.5 μ mol/L of each primer (Fig. 1), 10 μ mol/L of each molecular beacon probe, and 1 μ L of genomic DNA. The PCR program was run as follows: (1) denaturation at 95°C for 3 minutes and

(2) 40 cycles each of 1 minute at 95°C, 1 minute at 49°C, and 1 minute at 72°C. Fluorescent measurements were recorder during each annealing step with 2 different filter set, excitation/emission: FAM 492/516 and ROX 595/610.

RESULTS

Alleles Profile Detect by Sequencing

Comparative sequencing analysis showed that there was total concordance between genotyping results obtained with this molecular beacons real-time assay and with the PCR-DNA sequencing method used in this study (10 homozygous and 10 heterozygous M-Malton and 10 normal samples).

Melting Curve Analysis

By melting curve analysis using the MX 3000 real-time PCR system in the presence of the wild-type and the mutant synthetic oligonucleotide targets, the temperature ranges

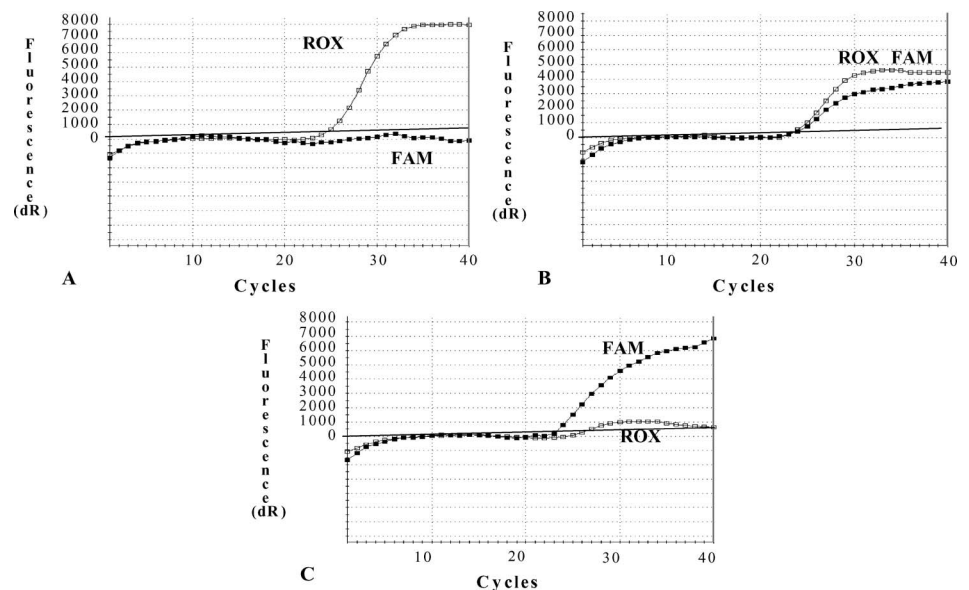


FIGURE 3. PCR real-time analysis with samples containing different M-Malton genotype profile: (A) homozygous mutated sample, (B) heterozygous, and (C) wild type.

allowing good allele discrimination can be drawn. As shown in Figure 2, at 45°C to 50°C temperature range, the wild-type molecular beacon 1 only hybridized with the wild type but not with mutant targets; thus, only wild-type targets produced a highest signal from the FAM channel. Likewise, the mutant molecular beacon 2 hybridized only with mutant target but not with wild-type sequences; thus, only mutant targets generated signal from the ROX channel (Fig. 2). At 49°C, the fluorescence difference between the complementary and non-complementary target level is evident and reliable for a PCR real-time detection. The denaturation analysis by melting curve indicates that displacing probes can recognize these allelic variations as well as the TTC deletion of 1 of the 2 consecutive 51/52 Phe codons.

Real-Time PCR Results

To evaluate the specificity of this method as a diagnostic tool for the determination of the homozygous or the heterozygous M-Malton ATD genotypes, a collection of clinical

cases was examined by conventional DNA sequencing and by real-time molecular beacon PCR. Chromosomal DNA was isolated from selected individuals carrying normal, homozygous, and heterozygous M-Malton genotypes. Data analysis was performed by analyzing the real-time amplification plots (Fig. 3) or with the MX 3000 software for alleles discrimination, which included the "Positive and Negative final call results" option, particularly useful for a clinical laboratory test (Table 1).

Patients' profiles, AAT serum concentration, sequencing analysis, and molecular beacon output, obtained using this novel PCR/real-time method, were analyzed and total concordance as well as good specificity and sensitivity were obtained (Table 1).

DISCUSSION

AATD is a hereditary disorder caused by several mutations in the AAT gene. Its major clinical manifestations include pulmonary emphysema in adults and liver disorders in

TABLE 1. Patients' Profile, Sequencing Results, and Molecular Beacon Output Analysis

Case No.	Age	Sex	Serum Level (mg/dL)	Clinical Symptoms	Sequence Analysis	Real-Time PCR	
						Rox	Fam
1.	43	M	<30	Lung disease	HM	+	-
2.	44	F	<30	Liver and lung disease	HM	+	-
3.	58	M	<30	Emphysema	HM	+	-
4.	2	M	<30	Liver disease	HM	+	-
5.	55	M	<30	Emphysema	HM	+	-
6.	10	F	<30	Liver and lung disease	HM	+	-
7.	35	M	<30	Liver disease	HM	+	-
8.	24	F	<30	Ealthy	HM	+	-
9.	50	M	<30	Emphysema	HM	+	-
10.	45	M	<30	Emphysema	HM	+	-
11.	57	F	80	Chronic hepatitis HCV+	H	+	+
12.	32	M	61	Hemorrhagic gastritis	H	+	+
13.	2	F	56	Increased serum GGT levels	H	+	+
14.	44	M	62	Chronic hepatitis HCV+	H	+	+
15.	5	F	74	Symptoms of liver disease	H	+	+
16.	14	M	73	Mononucleosis	H	+	+
17.	33	M	56	Symptoms of liver disease	H	+	+
18.	50	M	128	Symptoms of liver disease	H	+	+
19.	8	M	93	Increased serum GGT levels	H	+	+
20.	6	M	50	Increased serum GGT levels	H	+	+
21.	35	F	136	Ealthy	HW	-	+
22.	45	F	158	Ealthy	HW	-	+
23.	25	F	110	Ealthy	HW	-	+
24.	50	F	250	Ealthy	HW	-	+
25.	57	F	114	Ealthy	HW	-	+
26.	37	M	178	Ealthy	HW	-	+
27.	45	M	110	Ealthy	HW	-	+
28.	30	M	146	Ealthy	HW	-	+
29.	18	M	130	Ealthy	HW	-	+
30.	26	M	200	Ealthy	HW	-	+

Clinical symptoms: HCV, hepatitis C virus; GGT, gamma-glutamyl-transpeptidase.

Real-time PCR results are reported as positive and negative final call results: Rox, mutated molecular beacon sequence; Fam, wild-type molecular beacon sequence. Sequence analysis: HM, homozygous mutant; HW, homozygous wild type; H, heterozygous.

children as well as in adults. Because the nature of the symptoms depends on the mutation, an accurate diagnosis of the altered allele is critical for a possible preventive treatment.

The polymerase chain reaction has become an essential tool in the research and diagnostic laboratories and simple and reliable genotyping technology has been described. Nevertheless simpler, faster and more automated genotyping methods are needed for routine use and population studies, especially when only a small number of hot spots are focused.²⁶

Real-time PCR is now considered the PCR method with the best improved rapidity, sensitivity, and reproducibility and a reduced risk of carry-over contamination. Most of the popular real-time PCR chemistries depend upon the hybridization of a labeled oligoprobe, and, among different type of these probes, molecular beacons are increasingly being used in many applications involving nucleic acid mutation analysis. The stem-loop structure of molecular beacons provides in fact a competing reaction for probe-target hybridization that serves to increase probe sensitivity and specificity.^{27,28}

Therefore, the use of the real-time PCR, in particular in molecular diagnostics, has become one of the more interesting molecular assays, and this technology has been applied in different scientific areas like microbiology as well as studies of gene expression and genetic disease.

Here we report the development of a rapid qualitative fluorescent real-time PCR assay designed for the detection of the M-Malton AATD allele using 2 specific molecular beacons. M-Malton, also known as Mcagliari, is one of the 120 variants of the AAT serum protein. Only few of these AAT variants are associated to evident symptoms of liver or lung diseases and, even if the commonest pathologic alleles are considered, the Pi Z and the Pi S genotypes, also other rare variants, like the Mmalton allele, are involved in the development of AATD-related disorder. Several gene frequency studies suggest that an evident divergence exist between expected and diagnosed AATD cases, and novel diagnostic tools may raise this problem. There are many current methods available for PiZ and PiS genotyping, and a more useful multiplex real-time fluorescence test for these AATD alleles was recently described; nevertheless, only the direct sequencing of the AAT second exon is indicated for a correct detection of the M-Malton variant. For this reason, this multicolor Mmalton molecular beacons genotypic assay may contribute to improve the ATD diagnostic strategies. Homozygous and heterozygous M-Malton genotypes may be detected in real time in the same closed tube at multiple excitation and emission wavelengths. To fully realize the potential of molecular beacons, we have optimized their structure and the binding specificity of a molecular beacon-target duplex, using melting curves to establish the difference in the fraction of molecular beacon bound to wild-type target and that to mutant target. Optimization of the annealing/hybridization temperature, buffer composition and genomic DNA, primers, and probes concentration were also carefully tested. Clinical data and a comparative sequencing analysis of all the samples used in this study showed that this assay is

specific, easy to interpret, rapid, and reproducible. Therefore, this real-time PCR assay for the M-Malton genotyping, together with that already described for the PiZ and PiS variants, may contribute to the detection of a more complete range of AATD variants and therefore may be particularly useful in research and molecular diagnostics laboratories involved in the study of AATD-related diseases.

REFERENCES

1. Carrell RW, Lomas DA. Alpha 1-antitrypsin deficiency. *N Engl J Med*. 2002;346:45–53.
2. de Serres FJ. Worldwide racial and ethnic distribution of α_1 -antitrypsin deficiency. Summary of an analysis of published genetic epidemiology surveys. *Chest*. 2003;122:1818–1829.
3. Luisetti M, Seersholm N. Epidemiology of α_1 -antitrypsin deficiency. *Thorax*. 2004;59:164–169.
4. Jeppsson JO, Franzen B. Typing of genetic variants of α_1 -antitrypsin by electrofocusing. *Clin Chem*. 1982;28:219–225.
5. Ferrarotti I, Zorzetto M, Scabini R, et al. A novel method for rapid genotypic identification of alpha 1 antitrypsin variants. *Diagn Mol Pathol*. 2004;13:160–163.
6. Rodriguez F, Jardi R, Costa X, et al. Rapid screening for alpha 1-antitrypsin deficiency in patients with chronic obstructive pulmonary disease using dried blood specimens. *Am J Respir Crit Care Med*. 2002;166:814–817.
7. Ahsen N, Oellerich M, Schuz E. Use of two reported dyes without interference in a single tube rapid-cycle PCR: Alpha 1 antitrypsin genotyping by multiplex real-time fluorescent PCR with LightCycler. *Clin Chem*. 2000;46:156–161.
8. Brantly M. Alpha1-antitrypsin genotypes and phenotypes. In: Crystal RG, ed. *α_1 -Antitrypsin Deficiency*. New York: Marcel Dekker, Inc. 1996: 45–59.
9. Coni P, Pili E, Convertino G, et al. M_{varallo}: A new Mlike α_1 -antitrypsin-deficient allele. *Diagn Mol Pathol*. 2003;12:237–239.
10. Sergi C, Consalez GG, Faretti G, et al. Immunohistochemical and genetic characterization of Mcagliari α_1 -antitrypsin molecule. *Lab Invest*. 1994; 70:130–133.
11. Curiel DT, Holmes MD, Okayama H, et al. Molecular basis of the liver and lung disease associated with the α_1 -antitrypsin deficiency allele M-Malton. *J Biol Chem*. 1989;264:13938–13945.
12. Ferrarotti I, Baccheschi J, Zorzetto M, et al. Prevalence and phenotype of subjects carrying rare variants in the Italian registry for α_1 -antitrypsin deficiency. *J Med Genet*. 2005;42:282–287.
13. Stoller JK, Brantly M, Fleming LE, et al. Formation and current results of a patient-organized registry for α_1 -antitrypsin deficiency. *Chest*. 2000; 118:843–848.
14. McElvany NG, Stoller JK, Buist AS, et al, and the α_1 -antitrypsin Deficiency Registry Study Group. Baseline characteristics of enrollees in the National Heart, Lung and Blood Institute Registry of α_1 -antitrypsin deficiency. *Chest*. 1997;111:394–403.
15. Lomas DA, Mahadeva R. α_1 -Antitrypsin polymerization and the serpinopathies: pathobiology and prospects for therapy. *J Clin Invest*. 2002;110: 1585–1590.
16. Curiel DT, Holmes MD, Okayama H, et al. Molecular basis of the liver and lung disease associated with the α_1 -antitrypsin deficiency allele M-Malton. *J Biol Chem*. 1989;264:13938–13945.
17. Lodewyckx L, Vandevyver C, Vandervorst C, et al. Mutation detection in the alpha 1 antitrypsin gene (PI) using Denaturing Gradient Gel Electrophoresis. *Hum Mutat*. 2001;18:243–250.
18. Cox DW, Billingsley GD. Rare deficiency types of alpha 1 antitrypsin: Electrophoretic variation and DNA haplotypes. *Am J Hum Genet*. 1989; 44:844–854.
19. Graham A, Kalsheker NA, Newton CR, et al. Molecular characterisation of three alpha-1-antitrypsin deficiency variants: Proteinase inhibitor (Pi) nullcardiff (Asp256—Val); PiMmalton (Phe51—deletion) and PiI (Arg39—Cys). *Hum Genet*. 1989;84:55–58.
20. Marras SA, Kramer FR, Tyagi S. Multiplex detection of single-nucleotide variations using molecular beacons. *Genet Anal*. 1999;14:151–156.

21. Abravaya K, Huff J, Marshall R, et al. Molecular beacons as diagnostic tools: Technology and applications. *Clin Chem Lab Med.* 2003;41:468–474.
22. Sambrook J, Russell DW. *Molecular Cloning: A Laboratory Manual.* 3rd ed. Cold Spring Harbor, NY; Cold Spring Harbor Laboratory Press. 2001.
23. Walsh PS, Metzger DA, Higuchi R. Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. *Biotechniques.* 1991;10:506–510.
24. Zuker M. Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res.* 2003;31:3406–3415.
25. Monroe WT, Haselton FR. Molecular beacon sequence design algorithm. *Biotechniques.* 2003;34:68–72.
26. Kwok PY. High-throughput genotyping assay approaches. *Pharmacogenomics.* 2000;1:95–100.
27. Tsourkas A, Behlke MA, Rose SD, et al. Hybridization kinetics and thermodynamics of molecular beacons. *Nucleic Acids Res.* 2003;31:1319–1330.
28. Marras SA, Kramer FR, Tyagi S. Efficiencies of fluorescence resonance energy transfer and contact-mediated quenching in oligonucleotide probes. *Nucleic Acids Res.* 2002;30:e122.