Clinical Chemistry 47:4
739–744 (2001)

Automation and Analytical Techniques

Semiautomated DNA Mutation Analysis Using a Robotic Workstation and Molecular Beacons

Maarten L. Smit,[1] Belinda A.J. Giesendorf,[2] Jacqueline A.M. Vet,[1] Frans J.M. Trijbels,[1] and Henk J. Blom[1*]

Background: **Our increasing knowledge of the genetic basis of inheritable diseases requires the development of automated reliable methods for high-throughput analyses.**
Methods: **We investigated the combination of semiautomated DNA extraction from blood using a robotic workstation, followed by automated mutation detection using highly specific fluorescent DNA probes, so-called molecular beacons, which can discriminate between alleles with as little as one single-base mutation. We designed two molecular beacons, one recognizing the wild-type allele and the other the mutant allele, to determine genotypes in a single reaction. To evaluate this procedure, we examined the C677T mutation in the methylenetetrahydrofolate reductase (*MTHFR*) gene, which is associated with an increased risk for cardiovascular disease and neural tube defects. DNA was isolated from 10 μL of fresh EDTA-blood samples by use of a robotic workstation. The DNA samples were analyzed using molecular beacons as well as conventional methods.**
Results: **Both methods were compared, and no differences were found between outcomes of genotyping.**
Conclusions: **The described assay enables robust and automated extraction of DNA and analysis of up to 96 samples (10 μL of blood per sample) within 5 h. This is superior to conventional methods and makes it suitable for high-throughput analyses.**
© 2001 American Association for Clinical Chemistry

Our increasing knowledge of the genetic basis of disease has produced an exponential increase in the demand for molecular diagnostic analyses. Currently available techniques for mutation analysis are time-consuming and labor-intensive. To process large amounts of samples, the development of new, rapid, automated, and reliable assays is required.

The extraction of DNA from blood is the most labor-intensive and time-consuming procedure in DNA mutation analysis. Most extraction procedures require repeated pipetting, centrifugation, and incubation steps. In addition, this procedure is sensitive to human errors, e.g., sample interchange. Because of new DNA extraction procedures based on DNA-binding magnetic beads, centrifugation can be eliminated and implementation in robotic pipetting workstations can be performed. The use of a robotic pipetting workstation and magnetic beads *(1–3)* will not only reduce total handling and processing time, but will also eliminate the risk for sample interchange.

Amplification and detection of the isolated DNA usually is performed by PCR followed by restriction enzyme analysis and slab gel electrophoresis. A recently developed and very powerful technique for detection of DNA mutations is the molecular beacon technology *(4, 5)*. Molecular beacons are hairpin-shaped, fluorescent oligonucleotide probes that fluoresce only upon hybridization (Fig. 1). A molecular beacon consists of a loop that contains a probe sequence that is complementary to the target DNA molecule, and a stem formed by the annealing of complementary arm sequences located on either side of the loop sequence. A fluorophore is attached to the end of one arm and a quencher to the end of the other arm. The stem hybrid keeps the fluorophore and the quencher in close proximity, totally quenching the emitted fluorescence by dissipation of the energy as heat. In the presence of the complementary target, the molecular beacon will hybridize to the target and undergo a conformational change, forcing the fluorophore and quencher apart, allowing fluorescence to occur. Because of the hairpin structure, molecular beacons are more specific *(5–7)* than linear DNA probes and are therefore well suited for single-base pair mutational analysis *(8, 9)*. This technology is very fast because fluorescence can be measured during PCR, so-called real-time detection, and no post-PCR processing is necessary. Moreover, carryover contamination is eliminated because the fluorescence is measured through closed

University Medical Centre St. Radboud, [1] 424 Department of Paediatrics and [2] 564 Department of Clinical Chemistry CKCL, PO Box 9101, 6500 HB Nijmegen, The Netherlands.
*Author for correspondence. Fax 31-24-3618900; e-mail H.Blom@ckslkn.azn.nl.
Received September 13, 2000; accepted January 23, 2001.

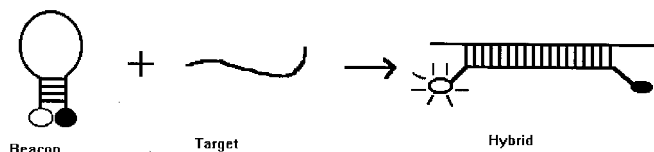

Fig. 1. Strategy of molecular beacons.

In the absence of the complementary target, the molecular beacon maintains its hairpin shape, keeping the fluorophore in very close proximity to the quencher. The quencher dissipates the fluorescence energy as heat, and no net fluorescence is measured. In the presence of the complementary target, the molecular beacon hybridizes to the target, forcing the fluorophore and quencher away from each other, and fluorescence occurs.

tubes. Those properties make molecular beacons well suited for automation of DNA mutation analysis.

In this report, we describe automated extraction of DNA from fresh EDTA whole blood by use of a robotic workstation followed by automated DNA mutation analysis using real-time PCR with molecular beacons as detector probes for mutation detection but not for quantitative purposes. To evaluate this procedure, we examined the C677T mutation in the methylenetetrahydrofolate reductase (*MTHFR*) gene, which plays an important role in homocysteine metabolism. A common mutation in this gene is the C677T mutation, which encodes an amino acid substitution (alanine to valine at position 226), producing a thermolabile MTHFR enzyme with decreased enzyme activity. This hampers remethylation of homocysteine to methionine, and homocysteine can thus accumulate, producing mild hyperhomocysteinemia. Mild hyperhomocysteinemia has been associated with an increased risk for the development of cardiovascular diseases *(10, 11)* and neural tube defects *(12)*.

To study the C677T mutation in the *MTHFR* gene, we modified our previously described molecular beacon assay *(8)*, which necessitated two reactions per sample. To increase the throughput of samples, we developed a multiplex detection assay using two differently colored molecular beacons. The molecular beacon labeled with the first fluorophore recognizes the wild-type allele, and the molecular beacon labeled with another fluorophore recognizes the mutant allele. Both molecular beacons were used in a single reaction. The fluorescence of both fluorophores was monitored in real time in a single tube to perform genotyping.

The reliability of the molecular beacon assay was determined by comparing its results with the results of the conventional technique.

Materials and Methods

SAMPLES

Fresh EDTA blood samples were collected from a random hospital population. No sample preparation was performed before extraction of DNA.

DNA ISOLATION USING A ROBOTIC WORKSTATION

DNA was extracted from 10 μL of whole blood using the Biomek 2000 (Beckman) robotic workstation with an integrated magnetic particle concentrator (Dynal A.S.) and magnetic beads (DNA Direct System I; Dynal A.S.) *(3)*. Blood samples were manually introduced into 96-well plates, of which four wells contained no blood to check for contamination during isolation. All additional steps were performed by the Biomek 2000. We used the lysis buffer, the washing buffer, and the resuspension buffer from the DNA Direct System I reagent set (Dynal A.S.).

The lysis buffer, consisting of a proprietary mixture of salts, detergents, and the magnetic beads, was added directly to all samples. In this step, lysis of the white blood cells takes place, releasing DNA, and the DNA instantly binds to the surface of the magnetic beads. The magnetic beads are spheres consisting of a nucleus of paramagnetic iron coated with a polymer to which the DNA can bind. Incubation took place for 5 min to allow the white blood cells to lyse and to form DNA-bead complexes. After the magnetic particle concentrator had been switched on, the supernatant was carefully removed and all samples were consecutively washed three times with washing buffer. The washing buffer was added vigorously in one action to wash the DNA-bead complex off the wall of the wells. The DNA-bead complex was recaptured after the magnetic particle concentrator had been switched on and the washing buffer was carefully removed. Finally, the DNA was resuspended by the addition of 50 μL of resuspension buffer (10 mmol/L Tris-HCl, pH 8.0), followed by repeated pipetting up and down to break the DNA-bead complex. After the DNA was resuspended, the magnetic particle concentrator was switched on again to capture the magnetic beads, which must be removed because they interfere in further analysis of the DNA. The resuspended DNA was automatically transferred to new 200-μL tubes for storage at −20 °C.

AMPLIFICATION AND ANALYSIS WITH MOLECULAR BEACONS

Extracted DNA (2 μL) was transferred automatically to the manually prepared PCR plate for real-time PCR. Three positive controls with known *MTHFR* genotype (wild-type, heterozygous, and homozygous mutant) and two additional negative controls were also used in every PCR run to check for contamination of the PCR mixture. The PCR mixture consisted of 250 μM dNTPs (Life Technologies), 1× PCR buffer (50 mM KCl, 10 mM Tris, pH 8.0; Perkin-Elmer), 4 mM $MgCl_2$ (Perkin-Elmer), 1.5 U of Taq Gold Polymerase (Perkin-Elmer), 0.4 pM forward primer BPF1 (Isogen), 0.4 pM reverse primer BPR1 (Isogen), and 50 ng each of C677T-wtTET and C677T-mutCFAM (Isogen) molecular beacons. Total reaction volume was 50 μL. PCR fragments of 143 bp were generated. The sequences of the primers and molecular beacons are listed in Table 1. The 96-well plate containing the DNA samples and PCR reaction mixture was manually transferred to the ABI Prism 7700 (Perkin-Elmer) for real-time PCR. Thermocycling conditions consisted of 10 min of initial denaturation at 95 °C to activate the TaqGold DNA polymerase, followed by 40 cycles of 30 s at 95 °C, 45 s at 56 °C,

Table 1. Sequences of primers and molecular beacons.

	Sequence[a]
Molecular beacons	
C677T-wtTET[b]	5′-TET-CGA GGT GCG GGA GC*C GAT TCC TCG-DABCYL-3′
C677T-mutCFAM	5′-FAM-CGA GGT GCG GGA GT*C GAT TCC TCG-DABCYL-3′
Primers	
BPF1 (forward)	5′-AAA GGC CAC CCC GAA GCA G-3′
BPR1 (reverse)	5′-ATG TCG GTG CAT GCC TTC AC-3′

[a] Underlined sequences indicate stem sequences; * indicates site of mutation.
[b] C677T-wtTET is the molecular beacon that recognizes the wild-type allele, and C677T-mutCFAM is the molecular beacon that recognizes the mutant allele.

and 30 s at 72 °C. Fluorescence data were collected at the annealing temperature of 56 °C with a laser exposure time of 100 ms. Before carrying out the experiments, we recorded the fluorescence spectra of both molecular beacons hybridized to an excess of their perfectly complementary target at 56 °C. Those spectra were stored in the computer as reference spectra, which were used by the computer to analyze the measured fluorescence spectra during the reaction.

Genotyping with molecular beacons was performed independently by two observers.

Determination of DNA quality after storage

To check the quality of the DNA samples extracted by the Biomek, samples were analyzed after storage at −20 °C for up to 8 months.

DNA amplification, restriction enzyme analysis, and agarose gel electrophoresis

Genotypes were also determined by conventional methods using PCR, followed by *Hin*fI restriction enzyme analysis and agarose gel electrophoresis as described by Frosst et al. *(13)*.

Determination of genotypes by conventional procedures was also performed by two independent observers.

Results

The aim of this study was to develop a semiautomated DNA analysis assay, consisting of DNA isolation using a robotic workstation and analysis using real-time PCR with molecular beacons as detection probes.

DNA isolation using a robotic workstation

Extraction of DNA from whole blood using a robotic workstation yielded uniform fragments of ~12 kb *(3)*. The total DNA yield was 100–200 ng, or a DNA concentration of 2–4 ng/μL of Tris-HCl buffer.

Amplification and analysis using molecular beacons

DNA samples, extracted from 10 μL of EDTA whole blood on a robotic workstation, were amplified and analyzed using real-time PCR with molecular beacons as detection probes. Fig. 2 shows the amplification plots of *MTHFR* wild-type, heterozygous, and homozygous mutant samples. To compare multiple amplification plots, we normalized the fluorescence for each molecular beacon, using the equation $(F - F_{min})/(F_{max} - F_{min})$, where F is the measured fluorescence, and F_{max} and F_{min} are the maximum and minimum fluorescence, respectively. In the top panel of Fig. 2, which shows the fluorescence results for a homozygous wild-type sample, the TET beacon, which recognizes the wild-type allele, clearly fluoresces, whereas the 6-carboxy-fluorescein (FAM) beacon, which recognizes the mutant allele, does not generate any fluorescent signal detectable above background. This indicates that there is no cross-hybridization of the mutant FAM beacon with the wild-type allele. In the middle panel of Fig. 2, both molecular beacons clearly fluoresce, which indicates the presence of both alleles, meaning that it is a heterozygous genotype. The bottom panel of Fig. 2 shows a homozygous mutant sample. In this case, only the beacon recognizing the mutant allele fluoresces, demonstrating the presence of the mutant allele and the absence of the wild-type allele.

The negative samples showed no fluorescence for either label, and the three positive control samples, containing the three different *MTHFR* genotypes, were correctly genotyped. None of the four instrument blanks from the DNA extraction procedure, which contained no template, generated any fluorescent signal, indicating that no contamination had occurred during DNA extraction by spilling or contaminated reagents.

In general, one or two samples per 96-well plate could not be genotyped by the molecular beacons because of loss of DNA during isolation or the presence of contaminants that inhibit PCR. Absence of DNA in a sample was demonstrated by agarose gel electrophoresis. Genotyping by molecular beacons and conventional methods generated identical results (data not shown). No differences in outcome of genotypes between the two observers occurred for both methods.

Stability of the isolated DNA

Repeated analyses of the DNA samples that were stored for 8 months at −20 °C produced the same unambiguously interpretable amplification plots as were generated with the freshly isolated DNA. This indicates that no degradation of the DNA had occurred during that period.

Discussion

In this report we describe a semiautomated method to perform DNA mutation analysis, consisting of automated extraction of DNA from whole blood with a robotic workstation, followed by analysis using molecular beacons in real-time PCR. We compared our semiautomated method with a conventional method using manual DNA isolation followed by PCR, restriction enzyme analysis, and slab gel electrophoresis. Other technologies have also

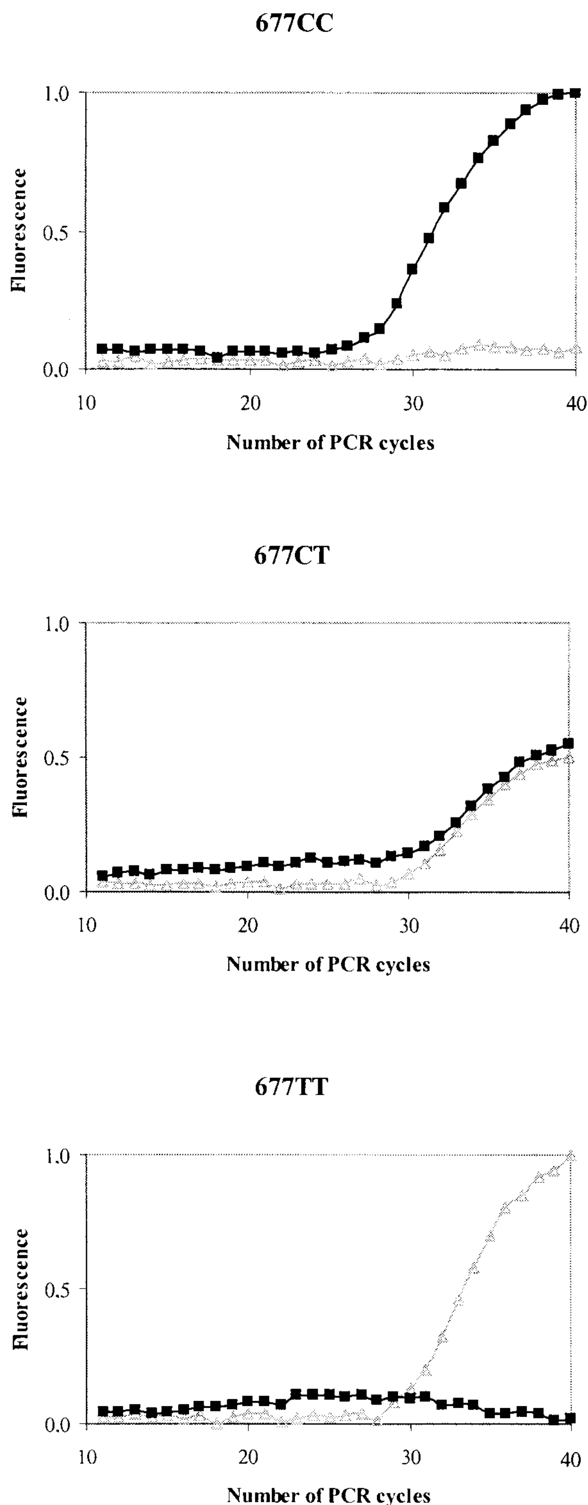

Fig. 2. Real-time amplification plots of three different genotypes: a homozygous wild-type individual (*top*), a heterozygous individual (*middle*), and a homozygous mutant sample containing the C677T mutation (*bottom*).

Normalized fluorescence of both the mutant beacon (△) and the wild-type beacon (■) is plotted on the *y-axis* and the number of PCR cycles on the *x-axis*.

been developed over the past few years to increase sample throughput, including robotic workstations to perform automated DNA extraction, e.g., the MagnaPure robotic system from Roche Molecular Systems and the BioRobot 9604 from Qiagen. The latter two instruments use methods based on binding of nucleic acids to membranes and elution using vacuum manifolds. The advantage of these methods is that more sample material can be processed than with magnetic beads, which increases the DNA yield up to 6 μg, according to the manufacturers. The disadvantages may be that commercial reagent sets are rather expensive.

To perform DNA mutation analysis, other real-time PCR technologies have been described recently in the literature. An alternative for molecular beacons is the use of an intercalating dye, such as Sybr Green I, which fluoresces only when bound to double-stranded DNA *(14, 15)*. PCR products can be visualized by the use of Sybr Green I and subsequently identified by their different melting behaviors after the PCR reaction. This method does not require any manual post-PCR processing, and assay costs are reduced compared with assays that use fluorescent hybridization probes. However, a disadvantage is the reliance on the design and optimization of the allele-specific primers and possible nonspecific amplification. The use of an intercalating dye also limits the possibility to perform multiplex genotype analysis.

Recently, a new technology has been described that uses amplicon-specific linear oligonucleotides (Roche Molecular Biochemicals) *(16, 17)*. Two differently labeled probes are used: one probe is labeled at the 3′ end with fluorescein, and the other probe is labeled at the 5′ end with a reporting dye. When both probes hybridize in close proximity on the target DNA, fluorescence resonance energy transfer occurs, producing a specific fluorescence emission of the reporting dye. Increasing the temperature during the fluorescence reading yields a fluorescence-vs-temperature curve, from which the melting point of the probe can be calculated. Under the appropriate conditions, the melting point of the probe will decrease in the presence of a mutation. In contrast to the use of an intercalating dye, this technology enables simultaneous measurement of independent targets by different fluorescent reporter dyes. Possible drawbacks of this assay are the requirement of two relatively expensive fluorescently labeled probes and the careful design of the assay.

In addition, new techniques that are not based on real-time PCR technology to perform semiautomated DNA mutation analysis have been developed, such as the ABI Prism 3700 DNA analyzer *(18)*, an instrument enabling high-throughput sequence analysis in a 96-well format. Another new sequencing method to analyze DNA fragments, recently described by Pyrosequencing Inc. (www.pyrosequencing.com/pages/technology.html), involves a technology that is based on generation of fluorescent signals by a cascade of different enzymatic reactions after incubation with a specific deoxynucleotide triphosphate.

An example of a high-throughput assay that is not based on fluorescent technology was described recently by Barbaux et al. *(19)*, who used a heteroduplex generator to simultaneously analyze more mutations. No restriction enzyme analysis was required after PCR, which substantially enhanced sample throughput. However, polyacrylamide gel electrophoresis was needed to analyze the PCR fragments. Another powerful technique to analyze DNA mutations, developed by Sequenom, is based on matrix-assisted laser desorption/ionization time-of-flight mass spectrometry *(20, 21)*, a technique that discriminates between DNA fragments by differences in molecular weight.

The goal of our experiment was to develop a semiautomated method for DNA extraction followed by mutation analysis of one single-nucleotide mismatch that does not require very high DNA yields. Automated DNA extraction using the Biomek 2000 and magnetic beads, followed by real-time analysis using molecular beacons, satisfactorily meets the requirements for analysis of known mutations. We adapted the general DNA isolation procedure using magnetic beads as described by the manufacturer for implementation in the robotic workstation. Our automated procedure of DNA extraction from small amounts of EDTA whole-blood samples using a robotic workstation and magnetic beads gave excellent results. In general, only one or two samples per 96-well plate could not be genotyped. This was caused by a loss of the DNA-bead complex during the extraction or by the presence of remnants of PCR inhibitors after the washing steps. A critical aspect was the removal of the magnetic beads from the final DNA resuspension, because magnetic beads interfere in fluorescence measurement by scattering the laser light in the ABI-Prism 7700. The DNA samples did not contain detectable amounts of beads and also were pure. No detectable degradation of the DNA by DNases, other impurities, or physical mechanisms had occurred during the 8 months of storage at −20 °C. However, some small degradation of the DNA samples would not negatively affect this particular assay. Advantages of this automated DNA extraction method are its reduced procedure time (96 samples within 2 h) compared with conventional manual methods, the requirement for very small amounts of blood (10 μL), and its full automation. The latter substantially reduces not only human labor, but also human errors. A minor disadvantage may be the lower yield of DNA (100–200 ng of DNA), mainly caused by the small sample volume of blood applied *(3)*.

In this study we analyzed a single nucleotide mutation, a C-to-T substitution at position 677 in the *MTHFR* gene. We used the property of enhanced specificity of molecular beacons compared with linear probes *(4, 6, 7)* to discriminate between alleles that differ by as little as one base. Two molecular beacons with different labels were designed, of which one was perfectly complementary to the wild-type allele and the other to the mutant allele. Both molecular beacons were used in a single reaction to double the throughput of the assay described previously by Giesendorf et al. *(8)*, who used only one molecular beacon per reaction. A second improvement compared with our previous study was the use of a redesigned molecular beacon that recognizes the mutant allele to minimize the occurrence of a G-T mismatch *(22)*. The beacon that recognizes the mutant allele carries a T at the putative site of mutation, which is able to form a relatively stable G-T mismatch with the complementary strand of the wild-type allele, which carries a G at the site of mutation. Therefore, the loop sequence of our molecular beacon was reduced from 15 to 14 bases, which improved specificity at the selected annealing temperature of 56 °C. Overall, genotyping using two molecular beacons in a single reaction gave excellent results. Unambiguously interpretable amplification plots were generated. No cross-hybridization occurred between the molecular beacon for the mutant allele and the wild-type allele. The instrument was also capable of distinguishing between fluorescent signals generated by the two different molecular beacons. The detection of wild-type and mutant alleles in one tube, using molecular beacons, was robust, and the outcomes of genotyping correlated completely with conventional data. In addition, all negative PCR controls and instrument blanks were negative, indicating that no contamination had occurred during the extraction procedure and preparation of the PCR mixture. Before the experiments described here, we investigated the occurrence of carryover contamination by running plates containing samples and blanks randomly distributed among the plate. Contamination was never observed in those studies (data not shown).

The combination of automated extraction of genomic DNA from whole blood using a robotic workstation with DNA analysis by PCR and molecular beacons produced a very fast procedure for DNA mutation analysis. DNA from up to 96 samples can be extracted and analyzed within 5 h, including PCR and mutation analysis, which is much faster than current conventional methods, which require ~2–3 days for the entire procedure. The risk of sample interchange is also strongly reduced and the risk for crossover contamination eliminated because no post-PCR processing is necessary. Because of their enhanced specificity compared with linear probes and the possibility to use a wide variety of fluorophores, molecular beacons are also suitable for simultaneous real-time analysis of more mutations in a single reaction *(9, 23)*.

At the moment, however, it is difficult to detect more than two mutations in a homogeneous solution with molecular beacons, mainly because of the limitations of different fluorophores that can be analyzed by the instruments as a result of spectral overlap and poor excitation by the laser. In addition to the ABI Prism 7700 we used in this experiment, new real-time PCR thermocyclers have been developed, such as the I-Cycler (Bio-Rad) and the LightCycler (Roche) *(24)*. The I-Cycler uses a conventional tungsten lamp as a light source in combination with different excitation and emission filters instead of a laser, which enables the use of a broader range of fluorophores

with less spectral overlap, making the development of multiplex assays much easier. The instrument also operates on a 96-well format. In the LightCycler, PCR reactions are performed in small capillaries, which allows faster heating and cooling rates, substantially reducing PCR time. Disadvantages are the limited capacity of 32 samples, which makes it incompatible with most robotic workstations that operate on a 96-well format, and the fact that only two colors can be measured simultaneously. The development of assays in which molecular beacons are hybridized to solid surfaces *(25–27)* may eventually enable simultaneous screening of all known mutations in genes involved in homocysteine metabolism and other inheritable disorders. Both the extraction of DNA and the hybridization of the DNA to the solid surfaces can then be performed by the Biomek 2000.

In conclusion, the assay described here enables reliable, rapid, and semiautomated DNA mutation analysis in hospital laboratories to perform large epidemiological studies and diagnostics.

This research was funded by the Noyons' Stichting, the European Demonstration Project Biomed BMH4-CT9505, and the Netherlands Heart Foundation (D97.021). We also gratefully acknowledge Annelies Mooren from the Central Clinical Laboratory CKCL for providing fresh EDTA blood samples.

References

1. Deggerdal A, Larsen F. Rapid isolation of PCR-ready DNA from blood, bone marrow and cultured cells based on paramagnetic beads. Biotechniques 1997;22:554–7.
2. Merel P, Dupin B, Comeau F, Lacoste L, Vezon G. Completely automated extraction of DNA from whole blood. Clin Chem 1996; 42:1285–6.
3. Smit ML, Giesendorf BA, Heil SG, Vet JA, Trijbels FJ, Blom HJ. Automated extraction and amplification of DNA from whole blood using a robotic workstation and an integrated thermocycler. Biotechnol Appl Biochem 2000;32:121–5.
4. Tyagi S, Kramer FR. Molecular beacons: probes that fluoresce upon hybridization. Nat Biotechnol 1996;14:303–8.
5. Tyagi S, Bratu DP, Kramer FR. Multicolor molecular beacons for allele discrimination. Nat Biotechnol 1998;16:49–53.
6. Bonnet G, Tyagi S, Libchaber A, Kramer FR. Thermodynamic basis of the enhanced specificity of structured DNA probes. Proc Natl Acad Sci U S A 1999;96:6171–6.
7. Tapp I, Malmberg L, Rennel E, Wik M, Syvanen AC. Homogeneous scoring of single-nucleotide polymorphisms: comparison of the 5′-nuclease TaqMan assay and Molecular Beacon probes. Biotechniques 2000;28:732–8.
8. Giesendorf BA, Vet JA, Tyagi S, Mensink EJ, Trijbels FJ, Blom HJ. Molecular beacons: a new approach for semiautomated mutation analysis. Clin Chem 1998;44:482–6.
9. Marras SA, Kramer FR, Tyagi S. Multiplex detection of single-nucleotide variations using molecular beacons. Genet Anal 1999; 14:151–6.
10. Kluijtmans LA, van den Heuvel LP, Boers GH, Frosst P, Stevens EM, van Oost BA, et al. Molecular genetic analysis in mild hyperhomocysteinemia: a common mutation in the methylenetetrahydrofolate reductase gene is a genetic risk factor for cardiovascular disease [see comments]. Am J Hum Genet 1996;58:35–41.
11. Kluijtmans LA, Kastelein JJ, Lindemans J, Boers GH, Heil SG, Bruschke AV, et al. Thermolabile methylenetetrahydrofolate reductase in coronary artery disease. Circulation 1997;96:2573–7.
12. van der Put NM, Steegers-Theunissen RP, Frosst P, Trijbels FJ, Eskes TK, van den Heuvel LP, et al. Mutated methylenetetrahydrofolate reductase as a risk factor for spina bifida [see comments]. Lancet 1995;346:1070–1.
13. Frosst P, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG, et al. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase [Letter]. Nat Genet 1995;10:111–3.
14. Press RD. Detection of prevalent genetic alterations predisposing to hemochromatosis and other common human diseases. Clin Chem 2000;46:1526–8.
15. Bon MA, van Oeveren-Dybicz A, van den Bergh FA. Genotyping of HLA-B27 by real-time PCR without hybridization probes. Clin Chem 2000;46:1000–2.
16. van den Bergh FA, van Oeveren-Dybicz AM, Bon MA. Rapid single-tube genotyping of the factor V Leiden and prothrombin mutations by real-time PCR using dual-color detection. Clin Chem 2000;46:1191–5.
17. von Ahsen N, Schutz E, Armstrong VW, Oellerich M. Rapid detection of prothrombotic mutations of prothrombin (G20210A), factor V (G1691A), and methylenetetrahydrofolate reductase (C677T) by real-time fluorescence PCR with the LightCycler. Clin Chem 1999;45:694–6.
18. Karger AE. Separation of DNA sequencing fragments using an automated capillary electrophoresis instrument. Electrophoresis 1996;17:144–51.
19. Barbaux S, Kluijtmans LA, Whitehead AS. Accurate and rapid "multiplex heteroduplexing" method for genotyping key enzymes involved in folate/homocysteine metabolism. Clin Chem 2000; 46:907–12.
20. Buetow KH, Edmonson M, MacDonald R, Clifford R, Yip P, Kelley J, et al. High-throughput development and characterization of a genomewide collection of gene-based single nucleotide polymorphism markers by chip-based matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Proc Natl Acad Sci U S A 2001;98:581–4.
21. Tang K, Fu DJ, Julien D, Braun A, Cantor CR, Koster H. Chip-based genotyping by mass spectrometry. Proc Natl Acad Sci U S A 1999;96:10016–20.
22. Ikuta S, Takagi K, Wallace RB, Itakura K. Dissociation kinetics of 19 base paired oligonucleotide-DNA duplexes containing different single mismatched base pairs. Nucleic Acids Res 1987;15:797–811.
23. Vet JA, Majithia AR, Marras SA, Tyagi S, Dube S, Poiesz BJ, Kramer FR. Multiplex detection of four pathogenic retroviruses using molecular beacons. Proc Natl Acad Sci U S A 1999;96: 6394–9.
24. Wittwer CT, Ririe KM, Andrew RV, David DA, Gundry RA, Balis UJ. The LightCycler: a microvolume multisample fluorimeter with rapid temperature control. Biotechniques 1997;22:176–81.
25. Liu X, Tan W. A fiber-optic evanescent wave DNA biosensor based on novel molecular beacons. Anal Chem 1999;71:5054–9.
26. Fang X, Liu X, Schuster S, Tan W. Designing a novel molecular beacon for surface-immobilized DNA hybridization studies. J Am Chem Soc 1999;121:2921–2.
27. Steemers FJ, Ferguson JA, Walt DR. Screening unlabeled DNA targets with randomly ordered fiber-optic gene arrays. Nat Biotechnol 2000;18:91–4.