precipitation step, which is identical in both methods. In contrast, in the MS method, such loss of analyte during sample preparation is fully compensated by the highly accurate principle of ID.

The ID-LC-TMS method described here is proposed for evaluation as a reference method for the quantification of 25-hydroxyvitamin D₃. The semiautomated sample preparation protocol and excellent practicability of LC-TMS allow the method to be used with large validation series, but the method is also applicable in routine laboratory settings. A multicenter validation of the method is currently planned with the goal of implementing an international reference system for 25-hydroxyvitamin D₃ measurement.

References

DOI: 10.1373/clinchem.2004.031831

Convenient Single-Nucleotide Polymorphism Typing from Whole Blood by Probe Extension and Bioluminescence Detection, Yukie Nakashima,1 Kazunori Okano,2 Kyoko Kojima,1 Hiromi Shirakura,3 Shinichi Ishida,3 Masaki Watanabe,4 Kohshi Maeda,4 Hiroyuki Tsujioka,2 Yasushi Imai,5 and Keiichi Nagai2 (1 Central Research Laboratory and 2 Advanced Research Laboratory, Hitachi Ltd., Tokyo, Japan; 3 Hitachi Science Systems Ltd., Tokyo, Japan; 4 Naka Division, Hitachi High-Technologies Corporation, Ibaraki, Japan; 5 Department of Cardiovascular Medicine, Graduate School of Medicine, the University of Tokyo, Tokyo, Japan; * address correspondence to this author at: Advanced Research Laboratory, Hitachi Ltd., Tokyo 185-8601, Japan; e-mail okano_kazunori@bxp.c.u-tokyo.ac.jp)

The information obtained on sequence variations in the genome in (1, 2) could make it possible for clinical science, based on molecular biology, to provide patient-specific medical care based on a patient’s genotype. Of these variations, single-nucleotide polymorphisms (SNPs) are the most common, and they are gradually being revealed as associated with phenotypes related to the onset or severity of diseases and to the effects of drugs. Methods for genotyping SNPs are becoming increasingly important for diagnosing diseases and determining an appropriate treatment for each patient.

We recently reported that SNP types can be determined by a new genotyping method based on the bioluminescence detection of the pyrophosphate produced in a specific probe-extension reaction. That method, called bioluminometric assay coupled with modified probe extension reactions (BAMPER) (3), requires that the single-stranded PCR products used as templates for probe-extension reactions be purified before the reaction by use of streptavidin-conjugated magnetic beads to remove the remaining PCR primers and substrates, which is a time-consuming and labor-intensive process. We also reported a BAMPER-based SNP-typing method that uses the PCR products directly and does not require single-stranded DNA (4). Before the SNP-typing probe-extension reaction, exonuclease I and shrimp alkaline phosphatase are used to remove the PCR primers, the residual deoxynucleotide triphosphates, and the residual pyrophosphate produced during the PCR reaction (5) to minimize the background intensity in the subsequent luciferase-luciferase assay. In this report we introduce a simplified and improved BAMPER method that can be used for SNP genotyping of whole blood drawn from individuals as well as for genotyping of purified genomic DNA by use blood dried on paper. The process flow for our method is shown schematically in Fig. 1 of the Data Supplement that
accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol50/issue8/.

The procedure for our novel BAMPER method is as follows. Whole-blood samples or purified genomic DNA were obtained from healthy volunteers in our laboratory. Written informed consent was obtained from every volunteer. A genomic region including a SNP site was amplified as PCR products from purified genomic DNA, whole blood, and blood dried on paper. A 100-μL aliquot of each blood sample was allowed to soak into a cellulose paper, which was then air-dried to immobilize the blood on the paper (6). For example, a fragment of the adrenergic β2 receptor gene (ADRB2) was first amplified as a 238-bp PCR product from purified genomic DNA (10 nmol), from 0.5 μL of blood (containing 7.5–15 nmol of genomic DNA), and from 0.5–0.75 μL of blood dried on the paper (containing 7.5–23 nmol of genomic DNA) by PCR (primers, 5′-AGC CTG CIG ACC AAG AAT AAG G-3′ and 5′-CCC TGG AGT AGA CGA AGA CCA T-3′). The amounts of PCR products obtained from the whole blood and dried blood were 25–30% (36–41 fmol/μL) of that obtained from the purified genomic DNA (136 fmol/μL).

Approximately 3 fmol/μL of the PCR product was required in the following SNP-typing steps. The other three genomic region including SNP sites were amplified and determined with the following primers: 5′-GCT CAT GCC CGT AAT CCT AGA-3′ and 5′-GGG AAA AAC CTT GCT TAT AAA-3′ for the gelatinase B gene; 5′-TTC CAC CTT CCT TTC TAC AGA-3′ and 5′-ACT AGG ATA TCG AAA TTG AAC AA-3′ for the Werner helicase gene (WRN); and 5′-GTC ATC CCT ATT GGC AGG TTA C-3′ and 5′-GCC AGG AGG GTC CGG TGA GAG T-3′ for the m ethylentetrahydrofolate reductase gene (MTHFR).

The SNP genotypes were determined by probe-extension reactions using two probes with different base termini: rs1042718 on the ADRB2 gene (5′-CCC ATT CAG ATG CAC TGG TCC N-3′, where N = A or C); rs3918242 on the gelatinase B gene (5′-CTC CCG AGT AGC TGG TAT TAT AGA CN-3′, where N = G or A); rs1346044 on the WRN gene (5′-CTC CTT TTG TTG ACA TCT CN-3′, where N = A or G); and rs1801133 on the MTHFR gene (5′-CTT GAA GGA GAA GGT GTC TGC GGG TGN-3′, where N = C or T). To prevent nonspecific extension of the probes and to improve hybridization accuracy, we replaced the third base sequences from the 3′ termini in the probes (underlined in above sequences) to provide a mismatch to the template DNA (3, 7). Pyrophosphate is produced during the extension reaction if the 3′ terminus of the specific probe is complementary to the SNP allele sequence. A Taq DNA polymerase was used for the allele-specific probe-extension reaction. A control reaction (without probes) and two extension reactions with two typing probes were carried out simultaneously. A reagent mixture containing 50 μM deoxy nucleotide triphosphate mixture (dCTP, dTTP, dGTP, and dATP-αS, treated with pyrophosphatase to remove contaminated pyrophosphate), 1.25 μM SNP genotyping probe (or control), and 0.138 U of Taq DNA polymerase was added to the PCR products. Each reaction mixture was then subjected to five shuttle cycles (94 °C for 10 s and 50 °C for 10 s), and then pyrophosphates were produced. The pyrophosphates were converted by pyruvate orthophosphate dikinase into ATP, which was detected in a luminescence assay (λ = 530 nm) using firefly luciferase (8).

We first demonstrated the reliability of our BAMPER method on three SNPs (rs3918242 for the gelatinase B gene, rs1346044 for the WRN gene, and rs1042718 for the ADRB2 gene), using hundreds of purified genomic DNA study samples. When data were converted to the ratio of the luminescence intensities obtained from two probes, the three groups of allele types were separated by more than the sum of 3σ of adjacent groups, indicating that we can set the region for judging the genotype as the mean ratio plus or minus 3σ and that the reliability of genotyping is higher than 99.7% (see Fig. 2 in the online Data Supplement). The high agreement between the results obtained with our improved BAMPER method and those obtained with the MassARRAY™ system (9) indicate the reliability of our genotyping method (see Table 1 in the online Data Supplement).

We next demonstrated the reliability of our BAMPER method, using three different starting materials, including blood dried on paper. For three individuals, the SNP genotypes at rs1042718 were analyzed from PCR products starting from the genomic DNA, whole blood, and blood dried on paper stored for 0, 8, and 15 days at room temperature (Fig. 1A). No amplified products were obtained from samples stored for 21 days. The genotypes of these three individuals were A/A, C/C, and A/C. The scores calculated from the ratios of the luminescence produced by probe extension to the luminescence attributable to nonspecific background reactions were grouped into three areas related to the three SNP genotypes, and consistent typing results were obtained from the genomic DNA, blood, and dried-blood samples. This indicates that SNP types can be determined from freshly sampled blood or from blood stored on paper for up to 15 days as accurately as they can be determined from genomic DNA. We also analyzed the SNP genotypes of 15 individuals, using samples of their blood dried on paper and stored for 15 days at room temperature. As shown in Fig. 1B, the typing scores were clearly scattered among two homozygotes and one heterozygote. The SNP genotypes obtained from the dried blood coincided with those obtained from the purified genomic as well as with those obtained from the MassARRAY system, the Invader® assay system (10), and DNA sequencing. Similar results were obtained for the other two SNPs (rs3918242 on the gelatinase B gene and rs1801133 on the MTHFR gene; see Fig. 3 in the online Data Supplement).

Our method, when used with blood dried on paper as a starting material, has two major advantages: It avoids the DNA extraction process, which is still too laborious, costly, and time-consuming for practical use in clinical
settings, even with many existing DNA-extraction reagents. In addition, it can also address a patient’s privacy protection concerns. In cases in which purified genomic DNA is used as the starting material for clinical diagnosis, because such DNA can be stored for many years, genetic information could easily be determined and disclosed without the consent of the individual long after the first testing. As we showed, however, the DNA in blood immobilized on paper does not remain intact after 21 days, which avoids the above problem.

In conclusion, we have developed a highly accurate SNP-typing method that uses only conventional instrumentation: a PCR instrument and a luminometer. The protocol is designed to yield SNP data in <4 h: 30 min for blood immobilization on paper, 2 h for PCR, 1 h for complete degradation of the PCR probes and substrates, 15 min for the probe-extension reaction, and 5 min for the luminescence assay. The data are obtained via addition of reagents through all four steps. Our genotyping method therefore has great potential for practical use in clinical settings as well as in biology laboratories.

We thank Kenko Uchida and Yoshinobu Kohara for excellent technical advice, comments, and discussions. We also thank Kiyoteru Noguchi and Tomoko Ogawa for support of this research. This work was performed as part of a research and development project of the Industrial Science and Technology Program supported by the New Energy and Industrial Technology Development Organization (NEDO) of Japan.

References
Amniotic fluid bilirubin peaks (1.6–1.8 mg/L) at ~19–22 weeks of gestation (1). An increase in amniotic fluid bilirubin concentration after 25 weeks indicates extensive hemolysis of fetal blood and potential erythroblastosis fetalis in which amniotic fluid bilirubin can reach ~10 mg/L (2, 3).

Bilirubin in amniotic fluid can be measured by direct spectrophotometry, most commonly by the “delta 450 bilirubin” method (4). Contaminating pigments, such as hemoglobin, can interfere in the method. Chloroform extraction has been used to extract bilirubin, leaving water-soluble pigments in the aqueous layer. The delta 450 bilirubin is then measured in the chloroform fraction and interpreted by use of the Liley plot (5, 6).

A delta 410 correction formula also exists that theoretically accounts for hemoglobin interference. The delta 410 absorbance is measured and used to correct the delta 450 bilirubin (4, 6, 7).

Our objective was to examine the effect of hemolyzed-whole-blood contamination on the delta 450 bilirubin measurement and to compare corrective methods.

After receiving Institutional Review Board approval, we pooled amniotic fluid specimens collected for physician-ordered fetal lung maturity assessment (stored at −20 °C). Visibly bloody or meconium-contaminated samples were not used. The pool was centrifuged at 1100 g for 5 min, and the supernatant was filtered through a 22 µm AP 20 prefiter (Millipore Corp.) and a 1.2 µm RA filter (Millipore). Bilirubin was undetectable in the pool by delta 450 measurement.

We prepared a 600 mg/L stock solution of unconjugated bilirubin (ICN Biomedicals Inc.) by dissolving 6.0 mg of bilirubin in 0.1 mL of dimethyl sulfoxide (Sigma Chemical Co.) and 0.2 mL of sodium carbonate (100 mmol/L). The solution was vortex-mixed until the bilirubin was dissolved. We then added 9.5 mL of pooled amniotic fluid, followed by 0.2 mL of HCl (100 mmol/L) to bring the pH to neutral. This solution was protected from light, poured into a foil-wrapped container, and stored at 4 °C.

Blood was collected from a volunteer donor into an EDTA tube. To lyse the erythrocytes, we subjected the blood to at least one freeze-thaw cycle at −20 °C. The total hemoglobin concentration and oxyhemoglobin and met-hemoglobin fractions, as measured on a blood gas analyzer, were 185 g/L, 89%, and 10% in the first experiment and 180 g/L, 79%, and 19% in the second.

The bilirubin solution was added to the pooled amniotic fluid to produce final bilirubin concentrations of 1.5, 4.5, 9, and 18 mg/L (corresponding to delta 450 bilirubin measurements of 0.076, 0.22, 0.43, and 0.82, respectively). To each of these groups, we added whole blood to produce hemoglobin concentrations of 0, 0.04, 0.2, and 0.4 g/L. Tubes were protected from light by use of aluminum foil. Samples were analyzed immediately on a Spectronic® Genesys spectrophotometer (Spectronic Instruments, Inc.) in quartz cuvettes with water as the reference unless otherwise indicated.

Amniotic fluid samples were scanned from 365 to 550 nm. The absorbances at 365, 450, and 550 nm were plotted vs wavelength on semilog paper. The baseline was drawn between the absorbances at 365 and 550 nm. The delta 450 was calculated as the difference between the absorbance and baseline at 450 nm (4).

After spectrophotometric scanning, the samples were mixed with an equal volume of spectrophotometry-grade chloroform (Sigma Chemical Co.) and vortex-mixed for 30 s. After centrifugation (1600g for 10 min at 2–4 °C), the chloroform layer was carefully removed and scanned in quartz cuvettes with chloroform as the reference (5).

For the recovery experiment, we used leftover amniotic fluid samples with no visible blood contamination that had been sent to the laboratory for physician-ordered delta 450 bilirubin measurements and had been wrapped in aluminum foil and frozen at −70 °C. Samples were thawed and mixed, and the delta 450 was measured immediately. After chloroform extraction, the delta 450 was measured immediately again.

The effects of bilirubin and hemoglobin on delta 450 measurements were estimated by ANOVA using the SAS Proc Mixed procedure (SAS Institute).

Addition of bilirubin up to 18 mg/L to amniotic fluid produced a linear increase in delta 450 bilirubin measurements (not shown). The effect of blood contamination on the delta 450 bilirubin measurement (Fig. 1A) was more pronounced (P = 0.0012) at lower bilirubin concentrations (1.5 mg/L; □). Hemoglobin decreased the absolute delta 450 bilirubin measurement from a baseline of 0.076 to 0.039 at 0.4 g/L hemoglobin. It was not until very high, pathologic concentrations of bilirubin (>9 mg/L; △) that the delta 450 bilirubin measurement, after the addition of blood, was within 10% of baseline.

Chloroform extraction reduced the effect of hemoglobin contamination (Fig. 1B), but decreased (P < 0.0001) the measured concentrations of bilirubin, even in samples with no hemoglobin added. At higher concentrations of