Mass Spectrometric Detection of an SNP Panel as an Internal Positive Control for Fetal DNA Analysis in Maternal Plasma

To the Editor:
Applications of fetal DNA detection in maternal plasma have been reported for the prenatal assessment of fetal RhD status, sex-linked disorders, and β-thalassemia. Because fetal DNA constitutes only 3% to 6% of the total DNA in maternal plasma (1), fetal sequences may occasionally go undetected because of low fetal DNA concentrations or fetal DNA loss during sample processing. Such false-negative results may lead to misinterpretation of the fetal genotype and consequently, false diagnoses. Thus the incorporation of analytical controls to confirm the presence of fetal DNA is recommended. Other investigators have developed a panel of insertion-deletion polymorphisms to serve this purpose (2). Single nucleotide polymorphisms (SNPs), however, are the most abundant class of polymorphisms in the human genome. We have recently developed a mass spectrometry based protocol, the single allele base extension reaction (SABER), that allows the sensitive and specific detection of fetal SNPs in maternal plasma (3). We applied SABER to develop an SNP panel to serve as an internal positive control for circulating fetal DNA detection.

Nine SNPs [see the Data Supplement that accompanies the online version of this letter at http://www.clinchem.org/content/vol53/issue1], each with a minor allele frequency of ≥35%, were selected from the RealSNP™ Assay Database (www.realsnp.com, Sequenom). For each SNP locus, primer extension assays based on the standard Homogenous MassEXTEND (hME) and the SABER principles were developed (3). The former was used to determine the fetal and maternal genotypes and the latter was for the detection of the fetal-specific SNP alleles from maternal plasma (3). The assay designs are summarized in the online Data Supplement. The extension products of all assays were then resolved and detected by the MassARRAY™ Analyzer Compact Mass Spectrometer (Bruker), a matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) system (4).

Forty-one women in the first trimester of pregnancy (gestational age range: 11–13 weeks) were recruited from the Prince of Wales Hospital, Hong Kong, with informed consent and institutional ethical approval. Peripheral blood (12 mL) was collected into EDTA tubes before chorionic villus sampling (CVS) and was processed to fractionate the plasma anduffy coat (3). DNA was extracted (4) and the maternal and fetal genotypes at each SNP were determined by the hME assays using maternal buffy coat and CVS tissues, respectively. A case would be considered informative if the mother was homozygous while the fetus was heterozygous for at least 1 SNP. This protocol was used because the paternally inherited fetal allele is most readily distinguishable from other maternal DNA molecules in maternal plasma because it is not possessed by the mother. If the SNP panel was adopted for clinical use, however, the fetal genotype would not be known at the time of analysis. Hence, SNP loci that would be applicable for any given case would be those for which the mother’s genotype was homozygous. For each of the applicable SNP loci, the SABER assay targeting the SNP allele not possessed by the mother would then be used for fetal DNA detection from maternal plasma. The positive detection of a unique SNP allele in maternal plasma but not the buffy coat fraction would suggest the presence of circulating fetal DNA.

In our use of this scheme, the number of SNP loci applicable to any 1 of the 41 fetomaternal pairs was 1 to 7. The number of applicable SNPs for which the fetus was heterozygous, i.e., informative, was 0 to 5. Overall, 90% of the cases were informative for at least 1 SNP from the panel. Maternal plasma fractions from half of the informative cases were used to optimize the SABER assays. Fetal allele detection from maternal plasma was then conducted blindly on the remaining 18 informative cases with

<table>
<thead>
<tr>
<th>Case</th>
<th>No. of applicable SNP loci from the panela</th>
<th>No. of informative SNPsb</th>
<th>No. of fetal informative SNPs detectable in maternal plasmae</th>
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<tr>
<td>57</td>
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<td>171</td>
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<tr>
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</tr>
<tr>
<td>881</td>
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</tr>
<tr>
<td>Total</td>
<td>74</td>
<td>41</td>
<td>36</td>
</tr>
</tbody>
</table>

a An SNP locus was considered applicable when maternal buffy coat analysis revealed a homozygous maternal genotype.

b An applicable SNP locus was further considered to be informative if CVS tissue analysis revealed that the fetal genotype was heterozygous.

c The number of informative fetal SNP loci detectable from maternal plasma by SABER analysis.

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the optimized SABER protocol (see the online Data Supplement). The fetal-specific allele was positively detected in 36 of the 41 SABER analyses in which the fetal genotype was informative (Table 1). When applied as a panel, the assays were able to detect the presence of the paternally inherited, fetal-specific allele in at least one of the informative SNPs in all of the cases (Table 1). There was no false-positive detection.

In summary, we have developed a panel of 9 SNPs to serve as positive controls for circulating fetal DNA. Additional SNPs could be added to increase the informative rate of the panel. Alternatively, placental epigenetic signatures could also serve such a purpose (5). Because the latter approach is not polymorphism based, it negates the need to determine the fetomaternal genotypes, but the epigenetic approach relies on bisulphite modification, which enhances the technical complexity of the analysis. The availability of both genetic and epigenetic fetal DNA controls would allow individual laboratories to choose the technology platform(s) that would be most easily implemented for their expertise.

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References


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Hemoglobin Constant Spring Can Interfere with Glycated Hemoglobin Measurements by Boronate Affinity Chromatography

To the Editor:

Hemoglobin (Hb) Constant Spring (CS) is a nondeleterious form of α-thalassemia that is most prevalent in southern Chinese and southeast Asian populations (1). The gene frequency ranges from 0.01 in central Asian populations (1), the frequency in Thailand, 0.033 in northern Thailand, 0.05 in Laos, to 0.05–0.06 in northeastern Thailand (2, 3). Elongated α chains of Hb are produced in low amounts and cause α-thalassemia. In the homozygous state, the effects of this variant are more severe than the corresponding conditions that result from α-globin gene deletion, and erythrocyte survival is markedly reduced (4).

Boron affinity chromatography measurement of glycated Hb is generally considered resistant to interference by most Hb variants including HbC and S trait (5, 6). For this reason we have adopted a boronate affinity chromatography method as our routine method of glycated hemoglobin measurement. Diabetic patient samples containing unusual variants are referred to our laboratory for glycated Hb testing when difficulties are encountered with cation exchange methods for HBAC.

We describe a case of a sample from a patient with HbE trait and HBCS for which measurement of glycated hemoglobin by boronate affinity chromatography was imprecise. Further investigation revealed that there was an extra peak in the chromatogram that interfered with proper integration of the glycated hemoglobin peak (Fig. 1A). This interfering peak was associated with the presence of HBCS in additional samples from patients without diabetes.

HBAC was measured with an automated boronate affinity chromatography method (Primus Corporation). Although the method measures total glycated Hb, it is calibrated to report results as HBAC equivalent values. Fructosamine was measured on a Modular P analyzer (Roche Diagnostics) with Roche reagents. All studies conducted using human samples were approved by the Institutional Review Board of the University of Utah.

The initial measurement gave a result of 8.9% HBAC, but because a shoulder was observed on the glycated hemoglobin peak, the sample was reanalyzed at the end of that same assay, giving a result of 7.0%. Based on these discrepant results, the sample was analyzed sequentially 10 additional times in the same assay. Results were 9.1%, 9.4%, 9.6%, 9.4%, 9.5%, 9.0%, 5.9%, 8.7%, 6.1%, and 8.9%, respectively. Inspection of the chromatograms revealed that for the results of 7.0%, 5.9%, and 6.1% peak integration was performed incorrectly due to the extra peak eluting between the nonglycated and glycated Hb peaks (Fig. 1A). For samples with HBAC results of ~9% the extra peak was integrated with the glycated Hb peak (Fig. 1B). The overall CV for 12 replicates of this sample was 15.8%. Excluding the 3 lowest
results gave a CV of 3.4%. A patient sample homozygous for HbA with an HbA1c of 8.2% was analyzed 10 times in 1 assay and gave a CV of 1.1%. The fructosamine concentration of a plasma sample from the index patient was 384 μmol/L (normal 285–328 μmol/L), consistent with an HbA1c result of 9%.

Phenotype analysis (HPLC, Variant analyzer, Bio-Rad Laboratories, beta-Thal short program) indicated 1.7% HbF, 4.6% P2 (HbA1c), 4.1% P3, 62% HbA0, 23% HbE, and 4.3% HbCS. HbCS eluted in 4 distinct peaks with retention times from 4.7–5.2 min. When we analyzed nondiabetic samples heterozygous for HbE (Fig. 1C) or containing HbCS (Fig. 1D), the sizes of the peaks eluting between nonglycated and glycated Hb were consistent with the concentration of HbCS in the samples. The presence of HbE did not produce such a peak.

The boronate affinity column that we used had a history of ~3500 injections. Analysis of the sample from the index patients and other samples heterozygous for HbE and HbCS on a column from a different lot with <700 injections demonstrated no peak between nonglycated and glycated Hb. Additional testing of samples heterozygous for HbE and HbCS on this column after 3300 and 4400 injections failed to demonstrate a shoulder on the glycated Hb peak, suggesting that this interference from HbCS is specific to one lot of columns and mobile phase.

In conclusion, the presence of HbCS in samples for glycohemoglobin analysis by boronate affinity chromatography has the potential to interfere with accurate measurement of glycated Hb. Any boronate affinity chromatogram with a shoulder on the glycated Hb peak should lead to careful review of how the peaks were integrated and repeat testing by the same or a different glycated Hb method.

We gratefully acknowledge the excellent technical support provided by the staff of the Automated Endocrinology Laboratory and the Hemoglobin Identification section of the Special Genetics Laboratory at ARUP Laboratories.

To Mix with Pooled Normal Plasma or Not to Mix: A Comparative Study of 2 Approaches for Assessing Lupus Anticoagulant Inhibitory Activity in the Dilute Russell Viper Venom Method

To the Editor:
The recommended (1) step-wise approach to the study of lupus anticoag-
uliant (LA) begins with a prolonged clotting time in a phospholipid-dependent clotting assay (screen), a non-correction in a mix with pooled normal plasma (PNP), and then a correction in the presence of increased phospholipid (confirm). Laboratories differ in their application of these recommendations. Jacobsen et al. (2) integrate screen, mix, and confirm into a single assay and analyze the data as a lupus ratio (LR). Tripodi et al. (3) propose no prior mix with PNP even for patients on oral anticoagulant (OAC) and analyze their data as a percentage correction. We wished to compare these 2 approaches by using the common (4) dilute Russell viper venom test (dRVVT) as the clotting assay.

Samples were collected from 500 consecutive patients (291 women, median age 56 years) for whom LA tests were requested during a 5-month period. Repeat tests (n = 19) were excluded on patients as were 9 samples that contained unfractious heparin. Of the remaining 491 patients, 85 were on OAC with interated heparin. Of the remaining 491 samples that contained unfractioned PNP even for patients on oral anticoagulant (OAC) and analyze their data as a percentage correction. We wished to compare these 2 approaches by using the common (4) dilute Russell viper venom test (dRVVT) as the clotting assay.

Plasma from 70 healthy volunteers (30 men and 40 women, median age 48 years) were used to establish the cutoff values. We used PNP from a pool of 30 similar healthy volunteers.

Blood from controls and patients was collected and stored as described (5). The LR of Jacobsen et al. (2) is the ratio of 2 clotting times for 1:1 mixes of patient’s plasma and PNP, one using a dilute phospholipid (screen) assay and the other using a phospholipid-rich reagent (confirm) assay. This ratio is normal-
ized by dividing by the corresponding ratio for PNP performed in the same assay. The percentage correction based on the study of Tripodi et al. (3) is the difference between the test plasma screen normalized by PNP screen and test plasma confirm also normalized by PNP confirm. This difference is expressed as a percentage of the normalized screen clotting time.

Clotting times for the same batch of PNP provided data for between-assay imprecision for over 20 assays (screen, 0.91%; confirm, 0.88%). The between-assay imprecision over 10 assays for LA-positive patient plasma was 1.4% for screen and 1.3% for confirm. The within-assay imprecision for PNP was 0.34% for screen and 0.27% for confirm (n = 12), and for the LA-positive patient plasma was 0.61% for screen and 0.59% for confirm.

For the dRVVT-based assays we used the La Screen and La Confirm reagents from Life Therapeutics. All tests were performed on an STA-R coagulation analyzer from Diagnostica Stago. We used the $\chi^2$ test to compare the LA positive pick-up rate in the LR and percentage correction assays. We constructed 2 × 2 tables to compare results derived from the LR and from the percentage correction.

Both LR and percentage correction for 70 individual normal individuals were normally distributed. The cutoff values (2 SD above the mean) for 70 normal individuals were LR = 1.08 and percentage correction = 13.5%.

For all patients, including the subset on OAC, the overall agreement of the 2 methods (LR and percentage correction) was 462/491 = 94%. The increased positive test rate for percentage correction (96/491 = 19.5%) compared with the LR (73/491 = 14.9%) was statistically significant at $P = 0.05$ (Table 1).

We subdivided the patient population into those on OAC (n = 85) and those not on OAC (n = 406). For patients on OAC, the concordance between the 2 methods was 74/85 = 87%. The increased positive test rate for percentage correction (23/85 = 27%) and LR (14/85 = 16.9%) did not differ significantly.

Samples from 26 patients (16 women, ages 19–76 years, median age 47 years) were LA positive by percentage correction and LA negative by LR (Table 1). The incidence of thrombotic disease (17 venous thromboembolic and 2 arterial thromboembolic) was high (73%) in this group. Six of these patients, all of whom were diagnosed with venous thromboembolic disease, were clearly positive with a percentage correction >3 SD (>18.1%) above the mean.

It has been reported (6, 7) that 1:1 mixes of test plasma and PNP may dilute a weak, yet potentially pathogenic LA, yielding a false-negative result. The high incidence of thrombotic disease in the 26 patients (9 of whom were on OAC) poses the question of whether LA activity was diluted by the 1:1 mix in these patients. The increased sensitivity of the percentage correction to the presence of a potential LA compared with the LR may well be of clinical value, warranting further studies to clearly characterize the presence of an antiphospholipid antibody in these patients.

### Table 1. Results of all 491 patients comparing the 2 methods of data analysis, dRVVT and percentage correction.

<table>
<thead>
<tr>
<th></th>
<th>Negative Test (&lt;13.53)</th>
<th>Positive Test (&gt;13.53)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative test</td>
<td>392</td>
<td>26</td>
<td>418</td>
</tr>
<tr>
<td>Positive test</td>
<td>3</td>
<td>70</td>
<td>73</td>
</tr>
<tr>
<td>Total</td>
<td>395</td>
<td>96</td>
<td>491</td>
</tr>
</tbody>
</table>

A direct comparison of the dRVVT LR and percentage correction shows an agreement of 462/491 (94.1%). Of the discrepant results (n = 29), 26 patients, 19 with clearly established thrombotic disease, were positive for LA by the percentage correction method only, and the remaining 3 patients with no evidence of thrombotic disease were positive by the LR method only. The percentage correction is significantly more sensitive to the presence of LA in the overall patient population ($P = 0.05$).

### References


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Many medical conditions are related to alterations in protein or hormone concentrations in blood. These changes are commonly detected with heterogeneous immunoassays that require a separation step before signal measurement. A simple homogeneous assay performed directly in whole blood would be useful in clinical diagnostics, but the performance of homogeneous fluorescence-based immunoassays has been severely limited by autofluorescence and strong absorption of ultraviolet and visible light in whole blood. These problems might be avoided by the use of near-infrared excited upconversion fluorescence. We recently described a novel homogeneous assay method based on upconversion fluorescence resonance energy transfer (FRET). In this method an upconverting phosphor (UCP) is used as a donor and a fluorescent protein or a small-molecular fluorescent dye is used as an acceptor (1, 2). We report here the use of a competitive homogeneous immunoassay for 17β-estradiol (E2) in whole blood as a model to demonstrate the application of upconversion FRET using a near-infrared fluorescent acceptor dye (Fig. 1A; and see Fig. 1 in the Data Supplement that accompanies the online version of this Letter at http://www.clinchem.org/content/vol53/issue1).

A UCP (Luminophor SPF) with a structural composition of La₄O₇:Yb⁺⁺,Er³⁺ was coated as described earlier (1–3) with an E2-specific recombinant antibody Fab fragment with a known cross-reactivity profile (4, 5). A Fab fragment was used to provide more favorable donor–acceptor distances for resonance energy transfer (5). The average size of the UCP donor particle was ~390 nm, determined with a Coulter N4 Plus submicron particle size analyzer (Beckman Coulter). A succinimidyl ester of a small-molecular acceptor dye, Alexa Fluor 680 (AF680) (Molecular Probes, Invitrogen Corp.), was coupled with a 2.5-fold molar excess of the amino-derivative of E2 (6-oxoestradiol 6-[O-(6-aminohexyl)oxime]) as described (2). Whole blood was collected in lithium heparin anticoagulated tubes (Venocject; Terumo Europe) from male volunteers at the Department of Biotechnology, University of Turku, and used in the assay with their informed consent.

To demonstrate the application of upconversion FRET using a near-infrared fluorescent acceptor dye (Fig. 1A; and see Fig. 1 in the Data Supplement), the anti-E2-Fab-coated FCD-546–1 UCP (donor) can bind E2 (analyte) and E2-AF680 conjugate (acceptor). Phosphor emission at 650–670 nm excites bound acceptors by FRET under the infrared excitation at 980 nm and sensitized acceptor emission can be measured at 740 nm. (B), standard curves in buffer (●, wide solid line), whole blood (■, dashed line) and plasma (▲, narrow solid line) were obtained after 45 min of incubation using 15 mg/L UCP donor and 4 nmol/L acceptor conjugate. IC₅₀ values (concentration that inhibited 50% of the maximum signal) of the assay were 0.72 nmol/L, 1.25 nmol/L, and 1.35 nmol/L, respectively. Standard curves were fitted to the data based on means of 4 replicates using the program Origin 6.0 (OriginLab Corporation) and the logistic function $y = \frac{A_1 - A_2}{1 + \left[ x/x_0 \right]^p} + A_2$, where $A_1$ and $A_2$ correspond to maximum and minimum values of the response, respectively, $p$ is the slope and $x_0$ is the IC₅₀ value. The error bars indicate the SD of the value, cts, counts.

Upconversion Fluorescence Enables Homogeneous Immunoassay in Whole Blood

To the Editor:

Many medical conditions are related to alterations in protein or hormone concentrations in blood. These changes are commonly detected with heterogeneous immunoassays that require a separation step before signal measurement. A simple homogeneous assay performed directly in whole blood would be useful in clinical diagnostics, but the performance of homogeneous fluorescence-based immunoassays has been severely limited by autofluorescence and strong absorption of ultraviolet and visible light in whole blood. These problems might be avoided by the use of near-infrared excited upconversion fluorescence. We recently described a novel homogeneous assay method based on upconversion fluorescence resonance energy transfer (FRET). In this method an upconverting phosphor (UCP) is used as a donor and a fluorescent protein or a small-molecular fluorescent dye is used as an acceptor (1, 2). We report here the use of a competitive homogeneous immunoassay for 17β-estradiol (E2) in whole blood as a model to demonstrate the application of upconversion FRET using a near-infrared fluorescent acceptor dye (Fig. 1A; and see Fig. 1 in the Data Supplement that accompanies the online version of this Letter at http://www.clinchem.org/content/vol53/issue1).
incubated at +30 °C by rotating the microtiter plate 6 times per minute around its horizontal axis to prevent erythrocyte sedimentation. Donor emission from the UCP and sensitized acceptor emission from the fluorescent dye were measured at 665 nm and at 740 nm, respectively, under continuous laser excitation at 980 nm and at 740 nm, respectively, unresonant dye were measured at 665 nm using Plate Chameleon (Hidex Oy) (3).

Examination of the ratio of maximum (without E2) and minimum (with 10 000 nmol/L E2) energy-transfer signals in buffer and whole blood with a 15 mg/L phosphor donor was optimal when 4 nmol/L of acceptor dye was used (see Fig. 2 in the online Data Supplement). Binding of the acceptor to the donor was even observed with a 31.25 pmol/L acceptor with a ratio of 8 in buffer and 6 in blood. Standard curves (Fig. 1B) and interassay precision profiles (see Fig. 3 in the online Data Supplement) for the E2-immunoassay, obtained with optimal donor and acceptor concentrations, were very similar in buffer, heparin plasma, and whole blood. Detection limits of the assay (defined here as concentrations that inhibited 20% of the maximum signal) in the 3 matrices were estimated to be 0.21 nmol/L (60 ng/mL), 0.46 nmol/L (120 ng/mL) and 0.49 nmol/L (130 ng/mL), respectively. Analytical recoveries for E2 in plasma and blood are presented in Table 1 of the online Data Supplement. Lower recoveries and higher detection limits in plasma and blood compared with buffer were assumed to result from the presence of endogenous steroid-binding proteins in biological matrices, increasing the total binder concentration in assay reactions (2). Minimum signals (background signals of the assay), originating from nonspecific binding interactions and nonproximity-based reabsorptive energy transfer (1, 2), were slightly increased in whole blood compared with other sample matrices, most likely because of stronger nonspecific binding interactions in blood.

Under infrared excitation, no autofluorescence from blood was detected at visible wavelengths, owing to the unique nature of upconversion. Because of the relative transparency of blood to light at wavelengths above 650 nm, the excitation of the UCP was not attenuated (see Fig. 4 in the online Data Supplement). The measurement of the sensitized acceptor emission above 700 nm completely eliminates the background arising from blood and from possible donor emission, even without temporal resolution in fluorescence detection. Moreover, in the upconversion FRET-based assay no emission from the acceptor alone is produced upon infrared excitation. The assay is relatively simple and can be performed in a standard microtiter plate assay format using uncomplicated detection instrumentation. The ability to perform homogeneous fluorescence-based immunoassays directly in whole blood provides new possibilities for biomedical research and clinical diagnostics.

This study was supported by the Finnish Funding Agency for Technology and Innovation (Tekes), the Academy of Finland (Grant No. 209417) and the Graduate School of In Vitro Diagnostics in Finland. A grant from the Instrumentarium Science Foundation denoted to K.K. is also gratefully acknowledged. The authors are thankful for technological support from Hidex Oy in anti-Stokes photoluminescence measurement and from Pirjo Laaksonen in drawing blood samples.

References

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A Novel Proximal −13914G>A Base Replacement in the Vicinity of the Common-13910T/C Lactase Gene Variation Results in an Atypical LightCycler Melting Curve in Testing with the MutaREAL Lactase Test

To the Editor:

The common −13910T/C exchange located 13910-bp upstream of the lactase gene (LCT) is associated with the persistence/nonpersistence trait of adult-type hypolactasia, which leads to symptoms of lactose malabsorption, including bloating, flatulence, diarrhea, and abdominal pain. The typing of this variant is used as a genetic test for lactase (non)persistence. We used the MutaReal Lactase real-time PCR test (Immunoagnostik) with the Roche LightCycler capillary system to analyze genomic DNA and observed in 1 sample an aberrant melting curve profile attributable to a novel base replacement, −13914G>A, in the vicinity of the common −13910T/C lactase gene variation.

Lactose intolerance results primarily from the physiological decline in activity of the LCT gene-encoded enzyme lactase-phorozin hydrolase, which breaks down milk sugar. Use of linkage disequilibrium and haplo-
type analysis of affected Finnish families revealed a defined DNA transition (−13910T/C) proximal to the LCT gene in intron 13 of the adjacent minichromosome maintenance type 6 gene (MCM6), which is associated with verified lactase nonpersistence and reduced bone mineral density predisposing for bone fractures (1, 2). Persons homozygous for C are at higher risk for lactose intolerance but may not show the typical symptoms. Nevertheless, genetic testing for the base-pair replacement is being offered as a genetic test for lactase persistence. The MutaREAL Lactase test is a real-time PCR for detection of this single-nucleotide polymorphism (SNP). A 222-bp PCR product is generated from the regulatory region of the lactase gene and analyzed by melting curve analysis. Typically, the genotype T/T has a Tm of 52 °C and the C/C variant of 61 °C with a variance of ±1 °C, and the heterozygous genotype has a biphasic melting curve with Tm,s at both temperatures.

According to the national guidelines approved by the German Medical Association, we routinely used the MutaREAL Lactase test to genotype patients who consulted the internal medicine or outpatient departments with symptoms of adult-type hypolactasia. In 121 samples, 26 patients (22%) were genotyped to T/T and 52 (43%) to C/T, and 42 (35%) were homozygote carriers of the C-allele associated with the lactose intolerance. This frequency, higher than the ~15%–20% in the general population in Germany (3), supports the linkage of this SNP with lactose intolerance, as established by specific experiments (4).

Genotyping of a 37-year-old male patient revealed an atypical, previously unreported melting curve (Fig. 1) displaying 2 peaks, with Tm,s at 52 °C and 57 °C. Although the Tm at 52 °C was typical for the T variant, the Tm at 57 °C represented neither the C nor the T variant. We performed standard PCR to amplify and sequence the 383-bp fragment spanning the corresponding region (initial denaturation at 94 °C for 3 min, then 35 cycles at 94 °C for 1 min, 57 °C for 1 min, 72 °C for 1 min, and final extension at 72 °C for 10 min) with primers LacU1 (5'-GAA TGC TCA TAC GAC CAT GG-3') and LacR1 (5'-CTG CTT TGG TTG AAG CGA AG-3'). This PCR generated the predicted fragment in all 3 controls (T/T, C/T, and C/C) and the patient sample (see Fig. 1 in the Data Supplement that accompanies the online version of this Letter at http://www.clinchem.org/content/vol53/issue1). Amplicons were purified, subjected to cycle sequencing with fluorescent dye terminators using primer LacU2 (5'-GGT AAG CAT TTG AGT GTA GTT G-3'), and analyzed on an ABI PRISM 310 automatic sequencer (PE Applied Biosystems). The analysis revealed that the patient was heterozygous for the −13910T/C variation and carried a previously unreported transition from G to A at position −13914 in one allele (see Fig. 2 in the online Data Supplement). We then cloned and sequenced respective allele fragments and found that the C at −13910 was linked to an A at position −13914 and the T at −13910 to a G at −13914 (see Fig. 3 in the online Data Supplement).

DNA fragments with the C and T variants in the −13910T/C region may enhance LCT promoter activity (5). The substitution at −13914 is not known to affect lactase activity, but the sequence 5'-GAT AAT GTA GC/T CCC TGG CCT C-3' surrounding the −13910 variant contains a binding site for an unidentified specific, differentially interacting, transactivation factor that can enhance lactase transcription in human intestinal cells (5). In addition, the −13910T/C variant is located in the MCM6 gene, which encodes a eukaryotic replication factor implicated and required for both initiation and elongation of chromosomal DNA replication. Further investigation of these variants may shed light on their clinical significance.

We report a genetic variation identified with fluorescent hybridization probes and melting analysis con-

![Fig. 1. Identification of a novel gene variation in the regulatory region of the lactase gene. The DNA melting curves resulting from the use of the MutaReal Lactase test to genotype the T/T, C/T, and C/C variants at the −13910T/C polymorphic sites in the lactase gene are shown. A novel variant leading to an arbitrary, biphasic melting curve with Tm,s at 52 °C and 57 °C was found in 1 patient.](image-url)
ducted on the LightCycler system. The increasing numbers of these reports suggest the need for an online database for aberrant melting curves and their underlying variations.

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Ex Vivo Simulation of Drug Action: Quantification of Drug-Induced mRNA as a Bridge Between Preclinical and Clinical Trials

To the Editor:

The recent tragedy, in which all active drug recipients developed life-threatening multiple organ failure during the phase I clinical trial of superagonistic anti-CD28 monoclonal antibody (TGN1412) (1), raised serious questions regarding the safety of human drug-trial participants. Results obtained in animal models do not always correspond to those observed in humans, and human cells placed in an in vitro culture environment may no longer exhibit physiologic functions that occur under natural conditions. An ideal precautionary method for investigational drugs designed to be administered intravenously is to mix each drug candidate with whole blood in test tubes and evaluate both desired function and unexpected adverse reactions. Such an ex vivo test is completely safe and applicable to drugs acting on leukocytes. The biggest technical challenge, however, is to identify drug actions in whole blood in a short period of time, because longer incubations might be associated with secondary artifacts. Gene expression analysis is an interesting candidate method, because mRNA transcription occurs earlier than protein synthesis and final biological outcomes.

In a recent issue of Clinical Chemistry (2), my group reported a novel assay system that quantified absolute amounts of mRNA in human whole blood by assessing the recovery of purified mRNA and the efficiency of cDNA synthesis in each sample. In this system, which used 50 µL of whole blood, small detectable variations among triplicate blood aliquots allowed us to identify increases as minute as 1.5–2-fold with statistical significance (2). Using this system, our group evaluated the effect of mouse antihuman CD28 monoclonal antibody because the humanized version used in the clinical trial (1) was not freely available.

We obtained anonymized blood samples from 6 healthy adult volunteers (Apex Research), after receiving institutional review board approval. Triplicate 60-µL aliquots of each heparinized whole blood sample were incubated with either 1 mg/L of mouse antihuman CD28 monoclonal antibody IgG1x or control purified mouse IgG1x (BioLegend) at 37 °C for 2 h. The concentrations of various mRNAs were quantified with SYBR green realtime PCR as described previously (2). Increases were calculated by the difference from control IgG. As shown in Fig. 1, after addition of mouse antihuman CD28 monoclonal antibody and incubation at 37 °C for only 2 h, whole blood from 1 healthy individual showed significant induction of inflammation- and apoptosis-related mRNA, such as interleukin-2 (IL-2), tumor necrosis factor superfamily (TNFSF)-1 (lymphotxin α), TNFSF-5 (CD40 ligand), CCL chemokine-8, CCL chemokine-20, and CXCL chemokine-10. The huge induction of IL-2 mRNA is reminiscent of the failure of systemic IL-2 therapy decades ago (3). In blood from other healthy individuals, we observed significant reductions in the concentrations of IL-6, TNFSF-2 (TNFα), CCL-8, CXCL-1, and CXCL-10 mRNA. The identification of these sensitive individuals serves as a warning of the potential toxicity of superagonistic anti-CD28 antibody as a drug candidate. The results also demonstrate that ex vivo mRNA analysis is an interesting assay model that can serve as a bridge from preclinical research to clinical trials.

Fig. 1. Drug-induced leukocyte mRNA in whole blood. Open circles (nonresponders) and open triangles (responder) indicate the mean values from each individual. * indicates the individuals with \( P < 0.05 \) between control and anti-CD28. Dashed box shows the range of 0.5–2 fold increase, at which the majority of responses were not significant.
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Antenatal Biochemical Expression of Cystinuria and Relation to Fetal Hyperechogenic Colon

To the Editor:
Cystinuria is an inherited disorder characterized by impaired apical transport of cystine and dibasic amino acids (e.g., ornithine, lysine, and arginine) in the renal proximal tubule and the small intestine epithelia. The overall estimated prevalence is 1/7000 neonates (1). Because of impaired renal cystine reabsorption, cystine precipitates and forms calculi that can produce urinary tract obstruction and may lead to renal insufficiency. Cystinuria is responsible for ~10% of all kidney stones observed in children; in ~50% of patients, stones form in the first decade of life. Diagnosis allows introduction of therapy to reduce stone formation and risk of renal impairment. Prenatal biochemical expression of the disease has not been described.

Fetal hyperechogenic small bowel (FHB), an infrequent ultrasound finding (0.1%–1.8% of pregnancies), is associated with severe fetal diseases such as cystic fibrosis and trisomy 21 (2). Even less common is fetal hyperechogenic colon (FHC) in which the hyperechogenicity is strictly limited to the colon. Recently, it has been suggested that FHC could be associated with cystinuria (3).

We found biochemical prenatal evidence of cystinuria and confirmed the association of cystinuria with FHC.

We retrospectively studied the clinical records of 782 pregnancies for which amniotic fluid (AF) was sent to our laboratory between January 2003 and May 2006 for biochemical investigation, because of digestive tract abnormalities (hyperechogenic bowel, dilated loops, and peritonitis) detected by routine ultrasound scan. Of them, 6 presented with FHC (Fig. 1) with normal AF digestive enzyme pattern. Thus, this study was restricted to the 3rd trimester of gestation and to AF with a normal digestive enzyme pattern (n = 197). We defined 3 groups: the 6 FHC cases (group 1), 12 FHB cases randomly selected from 175 (group 2), and a 3rd-trimester control group (group 3) consisting of 12 randomly selected AF samples from pregnancies followed for unrelated malformations detected at routine ultrasound scan. Informed consent was obtained for AF sampling in all cases. Samples were centrifuged (10 000 g, 5 min at 4 °C) and divided into 2 aliquots, 1 of which was immediately stored at −40 °C, while the other was assayed for digestive enzymes. The frozen aliquot was analyzed by ion-exchange amino acid chromatography using an Aminotac analyzer (Jeol) after deproteinization by 10-fold dilution in 200 g/L sulfosalicylic acid.

Concentrations of half-cystine, lysine, ornithine, and arginine in AF were within the previously
described reference intervals (4) for gestational age for groups 2 and 3, except for a low concentration of arginine found in 1 FHB patient. In contrast, these amino acid concentrations were all above the reference values in group 1, except for 1 patient with normal lysine and arginine values.

Chromosomal aneuploidy and cystic fibrosis were systematically excluded in the FHC group. The 6 FHC pregnancies went to term, and the newborns had no apparent complications. For 4 of these cases (including the patient with normal AF lysine and arginine), urine samples were obtained at 1–3 years of age. Cystinuria was confirmed by urine amino acid chromatography (half-life >10 times the upper limit of the reference interval), thus excluding transient hyperexcretion of cystine in the first months of life as an explanation (5).

Cystinuria is caused by defects in the amino acid transport system rBAT/b0,+AT of epithelial cells of the renal proximal tubule and small intestine (6). In the kidney, rBAT/b0,+AT is the main transport system for cystine reabsorption. During fetal life, tubular maturation begins after the 14th week. After 20 weeks, the kidneys provide >90% of the AF volume. Thus the AF amino acid chromatography in cystinuria-affected fetuses shows the same profile, as does postnatal urine.

The intestinal hyperexcretnicity in the 6 cystinuria-affected fetuses was not located in the small intestine, where the transporter is expressed, but downstream. During fetal life, the fetus continuously swallows AF. In the cystinuria-affected fetus, the renal reabsorption defect leads to an abnormally high concentration of cystine in AF. Because the rBAT/b0,+AT transport system is the only high-affinity system for cystine absorption in the small intestine, we propose that a progressive overload of the intestinal cystine transporter capacity occurs. Thus, unabsorbed cystine progressively concentrates and then precipitates in the intestinal tract. The occurrence of FHC after only 26 weeks can be explained by the progressive closure of the anal sphincter that commences after 22 weeks. Indeed, FHC resolved after evacuation of meconium in one of our cases.

These findings suggest that FHC should be studied as a potentially useful diagnostic indicator of cystinuria and could be regarded as a possibility to provide preventive medicine.

We thank all of the clinical staff who provided us with the detailed follow-up of patients and children, particularly Dr. Françoise Lloret (Isle-sur-la-Sorgue) and Prof. Florence Bretell (CH Nord Marseille). We are also indebted to all of the French Multidisciplinary Prenatal Diagnosis Centers that provided us with AF samples.

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To the Editor:

We have discovered that the DNA sample used in our recent paper (1) as a control heterozygote at the sickle cell locus of β-globin (17A→T, also known as HBB c.20A→T by HUGO nomenclature) contained an additional variant. Subsequent sequencing revealed a double heterozygote, HBB c.[9C→T; 20A→T]. The HBB c. 9C→T is a silent variant for the 3rd amino acid, histidine. In view of this additional sequence variation, we re-evaluated the heteroduplex scanning capabilities of the instruments, as reported in the original Fig. 3, to ascertain their ability to distinguish melting curves of heteroduplexes caused by single and double heterozygotes from melting curves of homoduplexes. The c. 9C→T is a common variant with an allele frequency of 38%, as determined in review of clinical samples submitted for β-globin sequencing (courtesy of Dr. Elaine Lyon, ARUP Laboratories).

The study was repeated as previously described (1), including both single and double heterozygotes. Eight instruments were evaluated for geno-
typing and heteroduplex scanning resolution (ABI’s 7000, Bio-Rad’s iCycler, Cepheid’s SmartCycler, Corbett’s Rotor-Gene 3000, Idaho Technology’s HR-1 and LightScanner, and Roche’s LightCycler 1.2 and LightCycler 2.0). The resulting normalized melting curves for genotyping and temperature-shifted curves of the 4 genotypes [wild-type, homozygous variant (c.20A→T), single heterozygote (c.20A→T), and double heterozygote (c.9C→T; 20A→T)] are shown in Fig. 1. The melting curves of the homozygous genotypes are similar to those reported earlier. Melting curves of heterozygous genotypes segregated according to the number of mismatches present, with the double heterozygote resulting in more low-temperature melting than the single heterozygote. After temperature shifting, the heterozygotes were readily distinguishable by curve shape (2–4).

The repeated analysis confirms our earlier results and further shows the capability of DNA melting analysis and its dependency on instrument resolution. Although all melting curves from heterozygotes are distinguishable, some resolve from the homozygotes with greater clarity than others after temperature correction (Fig. 1B), with the fundamental shape of each genotype differing from instrument to instrument. As seen by comparing the single heterozygous sample to the wild-type, the high-temperature regions merge on some instruments but are clearly distinct on other instruments. Heterozygous and homozygous samples segregate either by subtle changes in the melting curve slope or by the formation of multiple distinct melting features separated by inflection points, as was also seen in melting curves from the double heterozygote, for which some instruments resolved 2 melting regions and others 3. The continuing development of instruments with finer temperature control and fluorescence acquisition will lead to increased detail derived from heteroduplex and homoduplex contributions to the overall melting curve, providing even greater ability to identify unique sequence variants.

Aspects of melting analysis are covered by issued and pending patents owned by the University of Utah and licensed to Idaho Technology. C.T.W. holds equity interest in Idaho Technology.

Fig. 1. Melting curves of 110-bp HBB amplicon, including c.20A→T (sickle cell) and c.9C→T (silent variant). Each genotype was melted and displayed in triplicate on 8 different instruments. Melting curves for the homozygous wild-type are shown in green, the homozygous mutant (c.20A→T) in red, the single heterozygous mutant (c.[20A→T]) in black, and the double heterozygote mutant (c.[9C→T; 20A→T]) in blue. (A), normalized melting curves for genotyping; (B), temperature-shifted curves for heterozygote scanning.

References
Detection of Monoclonal Proteins by Capillary Zone Electrophoresis: Comparison of 2 Multichannel Automated Systems

To the Editor:

Capillary zone electrophoresis (CZE) is an alternative method for separation of serum proteins (1). Two dedicated and automated multichannel instruments are available, the Paragon 2000 (Beckman-Coulter) and the Capillarys (Sebia). The sensitivity and specificity of Paragon 2000 for detection of monoclonal proteins have been reported to be 95% (2–4) and 78% (4), respectively. The low reported specificity reflects the frequent occurrence of slight abnormalities in the electropherogram at the anodal part of the γ-globulin fraction (fibrinogen region) (4).

Only 2 studies (5, 6) evaluated the Capillarys for detection of monoclonal proteins and reported a high sensitivity, and prospective studies in a routine clinical setting are lacking.

We studied 597 consecutive samples submitted to the laboratory to test for the presence of a monoclonal protein or to reevaluate a known monoclonal protein. All samples were analyzed by the Capillarys (software version 5.2.1) and Paragon 2000 (software version 1.6.02) systems and by immunofixation, which was used as the reference standard. Immunofixation was performed on the Hydrasys Automa (Sebia) according to the manufacturer’s instructions, with the use of Hydragel 4 immunofixation gels.

Immunofixation revealed the presence of a distinct monoclonal protein in 246 of the 597 samples. The distribution of the types of monoclonal proteins is given in Table 1. The sensitivity of Capillarys for detecting a monoclonal protein was 90% [95% confidence interval (CI), 85%–93%]. This sensitivity was comparable to that of the Paragon, which was 91.5% (95% CI, 87%–95%; P = 0.5 by χ²).

In 267 samples, immunofixation revealed no abnormalities. The specificity of the Paragon was calculated to be 87% (95% CI, 83%–91%), higher than the specificity of the Paragon, which was 58% (95% CI, 52%–66%; P < 0.0001 by χ²). The lower specificity of the Paragon 2000 was attributable to the previously described (4) disturbed morphology at the fibrinogen position. If such disturbed morphology was not considered abnormal, then the specificity of the Paragon was 80% (95% CI, 75%–88%; P = 0.03 for comparison with Capillarys by χ²).

In 84 samples, immunofixation was difficult to interpret (presence of M-protein not excluded, M-protein very faint or not identifiable, differentiation between monoclonal and polyclonal pattern questionable). The results for the Paragon 2000 and Capillarys for these samples are available from the authors.

Quantification of the monoclonal protein by delimitation was possible in 87 samples. There was a good correlation between the Capillarys and Paragon (r = 0.99; Pearson). Bland-Altman analysis revealed an intercept of 0.81 (95% CI, 0.558–1.096) and a slope of 0.96 (95% CI, 0.95–0.98).

As shown above, each system may fail to detect monoclonal proteins. For example, the Paragon 2000 failed to detect a monoclonal protein in a sample in which immunofixation revealed an IgM monoclonal protein. This monoclonal protein was clearly detected by the Capillarys. Conversely, the Capillarys failed to detect a significant IgMκ monoclonal protein and an IgGκ monoclonal protein. In both samples the CZE electropherograms showed a minor disturbance in morphology of the γ-region but not the obvious monoclonal peak that was observed with agarose gel electrophoresis. A remarkable finding on the Capillarys analysis, observed in both samples, was an unusually long separation between the albumin fraction and the α1-globulin fraction.

We thank Sebia for providing the reagents to perform this study.

Table 1. CZE analysis of 246 consecutive samples positive by immunofixation.

<table>
<thead>
<tr>
<th>Immunoglobulin class</th>
<th>CZE, Abnormal Fraction Present, n</th>
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<tbody>
<tr>
<td></td>
<td>Paragon</td>
</tr>
<tr>
<td>IgA</td>
<td>34</td>
</tr>
<tr>
<td>IgD</td>
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<tr>
<td>λ</td>
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</tr>
<tr>
<td>Total</td>
<td>246</td>
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Prolonged Frozen Storage of Urine Reduces the Value of Albuminuria for Mortality Prediction

To the Editor:
Albuminuria is increasingly recognized as a cardiovascular risk factor in patients with diabetes and in the general population. Cardiovascular disease risk increases continuously with increasing urinary albumin excretion, starting at concentrations that once were considered healthy (1). We recently reported that, after prolonged frozen storage of urine samples, albumin concentrations may decrease, particularly those within the reference and microalbuminuric intervals (2). We also observed sample variation in the extent to which urinary albumin concentrations (UAC) decreased. In 2005, to investigate whether outcome predictions based on albuminuria were affected by assessment of UAC from frozen samples, we reassessed UAC in Prevention of Renal and Vascular ENd stage Disease (PREVEND) study baseline urine samples, collected and stored frozen at $-20^\circ C$ from 1997 to 1998. The PREVEND study was approved by the local medical ethics committee and conducted in accordance with the guidelines of the Declaration of Helsinki (3).

The PREVEND dataset was enriched for albuminuria. To create a representative sample of the general population, the enriched subset (UAC $>10$ mg/L) was reweighed by proportionally assigning an SPSS-generated random subset. Study participants with missing data on UAC and/or with leucocyturia, erythrocyturia, and/or kidney disease were excluded. Of the remaining 3249 persons, 104 died during follow-up. Power calculations indicated that 330 cases would provide a power of 90% with $\alpha = 0.05$ for detection of a difference of 0.05 in area under the ROC analyses between fresh and frozen samples. In the total PREVEND cohort, from 1997 to 1998 until December 2004, 340 persons died during follow-up. The dataset was enriched, therefore, with samples from persons who died, leading to a final study population of 3485 persons.

Handling of fresh and frozen urine samples, including hand inversions and centrifuging before analysis by nephelometry, was performed as described previously (2). The variability with calibration and reagent lot changes was 1.6% to 4.4% and 1.1% to 2.4%, respectively. The intra- and interassay CVs were 2.7% and 4.5%, respectively.

Participants in the PREVEND study gathered 2 urine samples on 2 consecutive days, allowing us to investigate whether prolonged frozen storage leads to additional error. We used the Pythagorean Theorem ($CV_{\text{total}}^2 = CV_{\text{analytical}}^2 + CV_{\text{biological}}^2 + CV_{\text{freezing}}^2$) to calculate $CV_{\text{freezing}}$ from the day-to-day variation in UAC for fresh and frozen samples. The median (interquartile range) UAC in fresh urine samples was 5.0 (3.3–8.6) mg/L. UACs were reassessed after a mean (SD) time period of 7 (0.4) y (range, 7.7–8.8 y) of frozen storage. After frozen storage, the mean (SD) percentage UAC concentration change was $-27$ (26)%, $-51$ (29)%, $-43$ (30)%, and $-14$ (17)% for samples with fresh UAC concentrations of $<10$, 10–20, 20–200, and $>200$ mg/L, respectively.

The between-day variations were 24%, 26%, 25%, and 16% for fresh samples ($CV_{\text{analytical}} + CV_{\text{biological}}$), and 27%, 40%, 37%, and 23% for frozen samples ($CV_{\text{total}}$) for the $<10$, 10–20, 20–200, and $>200$ mg/L categories, respectively. Freezing, therefore, introduced an additional variation ($CV_{\text{freezing}}$) of 12%, 30%, 27%, and 17% in the respective categories. The overall additional measurement error ($CV_{\text{freezing}}$) from frozen storage was 18% ($CV_{\text{analytical}} + CV_{\text{biological}}$ 25% and $CV_{\text{total}}$ 31%). ROC analysis revealed a mean (SE) area under the curve of 0.80 (0.014; $P < 0.001$) for the prediction of mortality by UAC assessed from fresh samples and 0.74 (0.016; $P < 0.001$) from frozen samples ($P = 0.006$ for comparison with fresh samples) (Fig. 1).

Our study is the first prospective report on a decrease in predictive properties of albuminuria for mortality after prolonged frozen storage of urine samples at $-20^\circ C$. We also found that freezing introduced additional measurement error along with a decrease in UAC. Introduction of an additional measurement error by freezing was suggested previously (2, 4), but to the best of our knowledge no previous studies actually compared frozen with fresh samples.

Based on the results of 2 previous studies, urine samples were more stable at $-70^\circ C$ than at $-20^\circ C$ storage (4, 5). In the 1st study, which investigated samples stored at $-20^\circ C$ and $-70^\circ C$ for 22 weeks, neither a significant decrease in UAC nor a significant difference between frozen storage at $-20^\circ C$ and $-70^\circ C$ was encountered. In the 2nd study, which assessed UAC only after frozen storage at $-20^\circ C$ and $-70^\circ C$ and not in fresh samples, significantly higher concentrations were found in samples stored at $-70^\circ C$ than at $-20^\circ C$. This study, however, did not investigate whether storage at $-70^\circ C$ indeed prevented a decline.

Our study shows that prolonged frozen storage of urine samples at $-20^\circ C$ adversely affects prediction of outcome by albuminuria. This effect is relevant for the interpretation and design of epidemiological stud-
ies, screening programs, and inter-
vention trials.

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