should also be acknowledged (or such individuals excluded) when establishing reference intervals. Consequently, our proposal is to establish reference intervals as a function of time of sampling to reveal the influence of time on reference limits for serum TSH. The outcome of sampling time investigations will indicate whether such data will lead to recommendations for time of sampling or to time-dependent reference intervals.

Studies to establish decision limits for serum TSH should be based on standardized measurements performed in longitudinal follow-up of cohorts with various concentrations of serum TSH. Such studies may well support the recommendation for serum TSH should be based on time-dependent reference intervals. Assembling measurement per-sampling time investigations will indicate the outcome of sampling to reveal the influence of time on reference limits for thyrotropin and thyroid hormones (1).

In a previous study (2) in which we divided UGT1A1 into 2 haplotype blocks (the 5’-flanking region and exon 1 in block 1 and common exons 2 to 5 in block 2), *60* and *IB* (perfectly linked 1813C>T, 1941C>G, and 2042C>G in the 3’-untranslated region in Japanese persons) showed increased total bilirubin concentrations in non-*28* patients. Because of the small number of patients, however, it was not clear whether bilirubin concentrations were affected by *60* and *IB* acting independently or cooperatively when they were on the same chromosome. To clarify this point, we reinvestigated the associations between the UGT1A1 haplotypes and total bilirubin concentrations in 554 healthy Japanese volunteers. The ethical review boards of the participating institutions approved this study, and informed consent was obtained from all participants.

For genotyping of *60*, *28*, *6*, and *IB* marker variations, DNA was extracted from Epstein-Barr-virus-transformed lymphoblastoid cells. The genotyping methods for the *60*, *6*, and *IB* alleles were described previously (3, 4). For *IB*, 1941C>G was genotyped (3). For *28*, −364C>T, which is perfectly linked with the *28* allele in Japanese persons (2), was used as a surrogate polymorphism, as described in Fig. 1 in the Data Supplement that accompanies the online version of this Letter at http://www.clinchem.org/content/vol53/issue2, which also shows the allele frequencies of the variations. The diploidy configuration (combination of haplotypes) for each volunteer was provided this study, and informed consent was obtained from all participants.

### References


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### A Combinatorial Haplotype of the UDP-Glucuronosyltransferase 1A1 Gene (*60*/*6B*) Increases Total Bilirubin Concentrations in Japanese Volunteers

**To the Editor:**

UDP-glucuronosyltransferases (UGTs) are a family of enzymes that glucuronidate many endogenous and exogenous substrates (1). Of the UGT1A gene isoforms, UGT1A1 is primarily responsible for glucuronidation of bilirubin (1). In east Asians, 2 well-known genetic variants, A(TA),TAA>A(TA),TAA (allele *28*, reduced transcription) and G71R (211G>A, allele *6*, reduced activity), are causative factors for increased plasma bilirubin concentrations in Gilbert syndrome (1). The *28* allele is almost always linked to the *60* allele (-3297T>G), with reduced in vitro transcription (2).

In a previous study (2) in which we divided UGT1A1 into 2 haplotype blocks (the 5’-flanking region and exon 1 in block 1 and common exons 2 to 5 in block 2), *60* and *IB* (perfectly linked 1813C>T, 1941C>G, and 2042C>G in the 3’-untranslated region in Japanese persons) showed increased total bilirubin concentrations in non-*28* patients. Because of the small number of patients, however, it was not clear whether bilirubin concentrations were affected by *60* and *IB* acting independently or cooperatively when they were on the same chromosome. To clarify this point, we reinvestigated the associations between the UGT1A1 haplotypes and total bilirubin concentrations in 554 healthy Japanese volunteers. The ethical review boards of the participating institutions approved this study, and informed consent was obtained from all participants.

For genotyping of *60*, *28*, *6*, and *IB* marker variations, DNA was extracted from Epstein-Barr-virus-transformed lymphoblastoid cells. The genotyping methods for the *60*, *6*, and *IB* alleles were described previously (3, 4). For *IB*, 1941C>G was genotyped (3). For *28*, −364C>T, which is perfectly linked with the *28* allele in Japanese persons (2), was used as a surrogate polymorphism, as described in Fig. 1 in the Data Supplement that accompanies the online version of this Letter at http://www.clinchem.org/content/vol53/issue2, which also shows the allele frequencies of the variations. The diploidy configuration (combination of haplotypes) for each volunteer was inferred by an expectation-maximization–based program, LDSUPPORT, as
described previously (2,5). Diplo- 
type configurations of the 521 volunteers 
without heterozygous *60 and *IB 
were obtained at 1.00 probability. Pre-
viously, we reported that UGT1A3
17A>G is linked with both *60 and *IB 
(5), and *IB is not linked with *28 and 
*6 (2). When UGT1A3 17A>G was 
included, all the diplotype configura-
tions were inferred with >0.95 proba-
ability.

To differentiate between allele 
and haplotype names, haplotypes 
are indicated by the * symbol plus 
the representative allele name. The 
haplotypes without marker varia-
tions were designated *1 for Block 1 
and *1A for Block 2 (2). Note that 
the *28 allele was perfectly linked 
with the *60 allele, but only half of 
the *60 allele, approximately, was 
linked with the *28 allele. Thus, the 
*28 haplotype harbors both *28 
and *60 alleles, whereas the *60 hap-
loype harbors only the *60 allele, as 
reported previously (2). The most 
frequent haplotype was *1-*IA (fre-
cquency, 0.545), followed by, in order, 
*6-*IA (0.079), *1-*IA (0.060), and 
*60-*IB (0.038).

We investigated the association of 
UGT1A1 haplotypes with total bi-
irubin concentrations (Fig. 1). P 
values <0.05 were considered signif-
icient. We used the Kruskal–Wallis 
test (P <0.0001) for statistical ana-
ysis of the differences in bilirubin 
concentrations among all diplo-
types, followed by the nonparamet-
ric Dunn multiple comparison test. 
Significant increases in bilirubin 
concentrations were observed in the 
*6-*IA/*6-*IA, *6-*IA/*6-*IA, *6-*IA/*60-
*IB, *60-*IA/*28-*IA, and *28-*IA/*28-
*IA volunteers compared with the 
*1-*IA/*1-*IA volunteers. An increasing 
trend (statistically not significant) 
in bilirubin concentrations (2.4-fold in-
crease) was seen in the two *60-*IB/
*28-*IA volunteers compared with the 
*1-*IA/*1-*IA volunteers. Significant 
increases in bilirubin concentrations 
have already been reported for *6 (*6-
*IA in this study) and *28 (*28-*IA) (1). 
Note that the median of total bilirubin 
values was not increased in the het-
erozygotes of *6-*IA/*1-*IA/*6-*IA and 
*28-*IA/*1-*IA/*28-*IA; Fig. 1).

We next analyzed the additive ef-
effects of *60-*IB and *6-*IA on *6-*IA 
and *28-*IA, respectively. A signifi-
cant increasing effect of *60-*IB on 
*6-*IA was observed for *6-*IA/*60-
*IB compared with *1-*IA/*6-*IA (P = 
0.0093; Mann–Whitney U-test). How-
ever, when *60-*IA/*28-*IA was com-
pared with *1-*IA/*28-*IA, the effect 
of *60-*IA was not statistically signif-
icant (P = 0.0513).

This study shows that either *60 or 
*IB alone has a slight effect on total 
bilirubin concentrations. The pres-
ence of both *60 and *IB on the same 
DNA strand (*60-*IB), however, sig-
nificantly increased bilirubin concen-
trations when present with *6-*IA on 
the other chromosome. Thus, at least 
in the Japanese population, *60 and 
*IB marker variations should also be 
incorporated into the UGT1A1 geno-
typing in addition to *6 and *28 
markers.

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Fig. 1. Association of the UGT1A1 diplotype with increased total bilirubin concentrations in 554 
Japanese healthy volunteers. 

Haplotypes are shown with * plus representative allele name. Note that haplotype *28 harbors both *28 and 
*60 alleles (see Fig. 1 in the online Data Supplement). Each point represents 1 volunteer, and the median 
is indicated by a horizontal bar. The Kruskal–Wallis test for the 21 diplotype yields a P value of <0.0001. 
Significant increases in bilirubin concentrations were detected in *6-*IA/*28-*IA (P <0.0001), *6-*IA/*6-*IA 
(P <0.0001), *6-*IA/*60-*IB (P = 0.0133), *60-*IA/*28-*IA (P = 0.0186), *28-*IA/*28-*IA (P = 0.0213), 
compared with *1-*IA/*1-*IA (nonparametric Dunnett multiple comparison test). a, P <0.05; b, P <0.0001.
Preanalytical Stability of Adrenocorticotropic Hormone Depends on Time to Centrifugation Rather than Temperature

To the Editor:

Preanalytical factors can affect reliability of hormone assay results. Adrenocorticotropic hormone (ACTH) in blood is considered highly unstable because of proteolytic degradation (1–4), so storage of blood samples on ice until analysis is recommended. In clinical practice, however, this procedure may present logistical problems because most samples for ACTH measurement must be shipped from the place of sample collection to the laboratory. Therefore, we studied the impact of time and temperature before plasma separation and analysis on the results of ACTH assays.

At 8 AM, we obtained 2 blood samples from each of 19 healthy volunteers and 2 patients with pathologically high ACTH values (1 with Addison disease and 1 with congenital adrenal hyperplasia). Volunteers and patients gave written informed consent, and the ethics committee for our institution approved the study. ACTH concentrations were 5–774 ng/L. As recommended by the manufacturer (Monovette, Sarstedt), collection tubes contained 1.2–2 g of potassium EDTA/L, with a maximum 1% dilution effect of liquid EDTA.

For each set of 2 samples, 1 sample was centrifuged immediately after collection and then divided into aliquots for storage at room temperature (22 °C), 4 °C, or –20 °C for 1, 2, 4, 24, or 48 h before being frozen at –80 °C until it was assayed. The 2nd sample was left in the primary collection tube at either room temperature or 4 °C for 1, 2, 4, 24, or 48 h before centrifugation and then frozen at –80 °C until it was assayed. All samples from 1 individual were analyzed in 1 run with an automated chemiluminescence assay (Advantage, Nichols). Results were compared with the concentration obtained from an aliquot stored under standard conditions (collected on ice, immediately centrifuged, and frozen at –80 °C until analysis) and expressed as percentage of standard condition.

We used pairwise 1-sided testing with the Wilcoxon signed-rank test to analyze the significance of changes in hormone concentrations. The duration of hormone stability was approximated by fitting a monexponentially decaying function to the raw data for each scenario and calculating the time period of 10% decreases in hormone concentrations compared with baseline concentrations under standard conditions. Analytical testing and curve fitting were implemented in Mathematica version 5 (Wolfram Research).

As expected, measured ACTH concentrations significantly decreased with time before freezing at –80 °C. Interestingly, temperature alone did not appear to influence hormone concentration stability (P > 0.05). The calculated times for decay of mean concentrations to 90% of baseline values at 4 °C and room temperature, respectively, were 24 h and 19 h for uncentrifuged samples and 33 h and 31 h for immediately centrifuged samples.

After 2 h of storage at 4 °C, the ACTH concentration was significantly higher in samples centrifuged immediately than in uncentrifuged samples (P < 0.01) (Fig. 1A). At 22 °C this difference was observed after 1 h (P < 0.05) (Fig. 1B). The decrease in the measured ACTH concentration with time before centrifugation was also observed in samples containing

![Fig. 1. ACTH concentrations drop significantly faster if samples are not centrifuged after venipuncture (mean (SE)); temperature per se is less important.](image-url)