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 384/9262 mol/L (normal 11021 <285/9262 mol/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 & E), 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.

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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-
ulan (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 unfractoned heparin. Of the remaining 491 patients, 85 were on OAC with international normalized ratios ranging from 1.7 to 3.5.

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 mixtures 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 normalized 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 imprecison for over 20 assays (screen, 0.91%; confirm, 0.88%). The between-assay imprecison over 10 assays for LA-positive patient plasma was 1.4% for screen and 1.3% for confirm. The within-assay imprecison 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 χ² 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.](image)

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**Letters**

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).

A UCP (Luminophor SPF) with a structural composition of La4O5:Yb3+,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 (Venocjet; Terumo Europe) from male volunteers at the Department of Biotechnology, University of Turku, and used in the assay with their informed consent. The E2 immunoassay was carried out in buffer, plasma, and blood according to an assay principle previously described (2). Blood and plasma samples were diluted in buffer to comprise 20% of the total 45-μL reaction volume. The reactions were

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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).

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