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# Evaluation of a Dried Blood Spot Assay to Measure Prenatal Screening Markers Pregnancy-Associated Plasma Protein A and Free β-Subunit of Human Chorionic Gonadotropin

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BACKGROUND: First-trimester prenatal screening for aneuploidy by use of dried blood spots (DBSs) may offer practical benefits in settings where the instability of intact human chorionic gonadotropin (hCG) is problematic. We evaluated a DBS pregnancy-associated plasma protein A (PAPP-A) and free β-subunit of hCG (free hCGβ) dual assay and compared it to serum screening.

METHODS: Hematocrit-corrected DBS PAPP-A and free-hCGβ concentrations were measured and compared with serum concentrations in 252 first-trimester samples. Serum intact hCG was also measured and, with serum free hCGβ, was used to fit a model to predict serum-equivalent DBS free-hCGβ concentrations. In a separate experiment, we investigated the effects of temperature and relative humidity during the blood spot drying process.

RESULTS: The DBS assay for PAPP-A performed similarly to the serum assay, whereas free-hCGβ DBS measurements were consistently higher than in serum. Purifying blood spots of intact hCG suggested that the free-hCGβ DBS assay is measuring a composite of free hCGβ and additional β-subunits from intact hCG. The drying experiment showed that increased temperature and relative humidity during the drying process resulted in increased free hCGβ and reduced PAPP-A.

CONCLUSIONS: Despite measuring additional free hCGβ compared to the serum assay, DBS analysis has a role in first-trimester combined screening for trisomy 21.



The first-trimester combined test for trisomy 21 and other aneuploidy measures pregnancy-associated plasma protein A (PAPP-A)[4] and free β-subunit of human chorionic gonadotropin (free hCGβ) in maternal serum and fetal nuchal translucency by ultrasound. These measurements are combined in a screening algorithm to produce likelihood ratios, which are multiplied by a priori maternal age–related risks, resulting in individual pregnancy-specific risks *(1, 2)*. This panel of tests has been demonstrated to have detection rates of ≥90%, with false-positive rates of 5% *(3–5)*.

Intact hCG consists of noncovalently bound α- and β-subunits and circulates in maternal blood at considerably higher concentrations than free hCGβ *(6)*. It has been shown that storage of serum and whole-blood samples at or above room temperature for prolonged periods of time results in increased free hCGβ, as new free β-subunits dissociate from the intact hCG molecule *(7, 8)*. In our own laboratory in the UK, a moderate association exists between local surface air temperature and free-hCGβ values, expressed as multiples of the median (MoM) euploid concentrations (median MoM = 0.96 + (0.00217 × temperature in degrees Celsius); $R^2 = 0.082$; $P < 0.001$). In warmer climates, such as parts of India, seasonal variations in monthly free-hCGβ medians are more marked, resulting in inaccurate risks being reported for women screened in the summer months when the median MoM can exceed 1.25 (personal communication, PerkinElmer India Private Limited, Mumbai, India). For example, at a risk cutoff of 1:200, a +20% deviation in median free-hCGβ MoM will result in an undesirable increase in the false-positive rate from 4.5% to 6.1% *(9)*.

Dried blood spot (DBS) sampling offers an alternative to separating and sampling serum. Whole blood from a skin prick or a blood collection tube is spotted onto filter paper and left to dry. Uniformly sized circles

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[4] Nonstandard abbreviations: PAPP-A, pregnancy-associated plasma protein A; hCGβ, β-subunit human chorionic gonadotropin; MoM, multiple of median; DBS, dried blood spot; SURUSS, Serum Urine and Ultrasound Screening Study; EQA, external quality assessment.

are then punched out into individual reaction vessels, along with buffers that elute the analytes of interest for measurement. The use of DBSs is routine in the newborn screening of conditions including phenylketonuria, hypothyroidism, and cystic fibrosis *(10)*. A first-trimester aneuploidy screening program that uses a PAPP-A and free-hCG$\beta$ DBS dual analyte method produced by NTD Labs (a PerkinElmer company) has been in clinical use for >15 years *(11)* and has recently been transferred to the AutoDELFIA (PerkinElmer) *(12)*. Furthermore, once the blood spots have dried, free hCG$\beta$ concentrations in DBSs do not appear to increase at room temperature and above *(13)*.

In this study, we set out to evaluate the PAPP-A and free-hCG$\beta$ AutoDELFIA DBS dual assay and compare it with the corresponding AutoDELFIA serum-based assays. In addition, we aimed to investigate the effects of extreme temperature and relative humidity during the blood spot drying process on the measurement of PAPP-A and free hCG$\beta$.

## Materials and Methods

### SAMPLE COLLECTION

Pregnant women attending the routine first-trimester prenatal screening clinic at King George Hospital (Goodmayes, Essex, UK) have blood samples taken into red-top Vacuette tubes (456089, Greiner Bio-One). The isolated serum is analyzed for PAPP-A and free hCG$\beta$ on the Kryptor system (Brahms). For the 252 women who enrolled in the present study in December 2010 and January 2011, within 60 s of venipuncture and before the blood had clotted, the cannula of a DIFF-SAFE blood dispenser (Alpha Scientific) was inserted into the rubber stopper of the Vacuette, the tube was inverted, and four blood drops were spotted onto Whatman 903 Protein Saver collection cards (Whatman), as demonstrated in an instructional video (PerkinElmer). Each card was dried overnight at laboratory room temperature (20 °C–24 °C). Immediately after spotting, the DIFF-SAFE device was removed, and the tube was returned for routine analysis, after which an aliquot of the serum was taken and frozen at −20 °C for later analysis in this study. The use of excess material for research is permitted by our local ethics committee.

### DBS ANALYTE MEASUREMENT

We analyzed DBSs for PAPP-A and free hCG$\beta$ with the AutoDELFIA PAPP-A/Free hCG$\beta$ dual assay (kit reference B027-105) on the day after collection. Disks (3.2 mm diameter) were punched out of the DBS with a DBS puncher (reference 1296-071, PerkinElmer) into individual wells of a 96-well microtiter kit. Blood was eluted from the DBS by kit buffers, as previously described *(13)*. We ran 3 levels of kit quality controls, provided as dried whole-blood spots, in duplicate (punched from the same spot) with each run, which were also used to calculate within-spot within-run and between-run CVs.

### INTACT hCG PURIFICATION

A preliminary study had indicated that free-hCG$\beta$ concentrations measured from DBSs were higher than expected. To investigate this additional free hCG$\beta$, we prepared an anti-hCG affinity column by coupling anti-hCG $\alpha$-subunit–specific antibodies (Medix), 15 mg protein per 1.7 mL gel, onto cyanogen bromide–activated Sepharose (GE Healthcare) according to the manufacturer's instructions. A first-trimester serum pool [excess serum from a screening program at Oulu University Hospital (Oulu, Finland), thawed from −20 °C], was run through the anti-hCG column to produce purified (intact hCG–free) serum. We measured serum PAPP-A, free-hCG$\beta$, and intact-hCG concentrations before and after purification. Both intact hCG–free and nonpurified sera were mixed with washed red blood cells and used to prepare blood spots, which were dried and then measured for PAPP-A and free hCG$\beta$.

### COMPARISON OF DBS AND SERUM

The serum samples corresponding to the DBSs from the main study were thawed and analyzed on the AutoDELFIA for PAPP-A (kit reference B098-201) and free hCG$\beta$ (kit reference B097-101), as well as for intact hCG (kit reference B082-101), which was measured as a potential explanatory variable for predicting DBS free-hCG$\beta$ concentrations. We measured hemoglobin concentrations with the Sysmex Analyser (Sysmex UK) on EDTA-anticoagulated whole-blood samples collected at the same time as the screening sample, retrievable from a central database in 203 (81%) of the patients. We used the sample median hemoglobin concentration in the missing cases. Patient percentage hematocrit was estimated by multiplying the hemoglobin concentration (in grams per deciliter) by 3 *(14)*.

### DRYING EXPERIMENT

We prepared 8 blood sample pools by mixing washed red blood cells with pools of first-trimester serum (from Oulu University Hospital). We chose 12 combinations of temperature and relative humidity across the ranges of 20 °C–37 °C and 10%–80% relative humidity over a period of 3 days in 3 environmentally controlled chambers (Binder). For each drying condition, 1 Whatman 903 filter paper cassette was used per blood sample, and 5 50-$\mu$L spots of blood were spotted per cassette. Immediately after spotting, the cassettes were placed into the drying chamber for 3 h. Two disks

were punched out of each of the 5 spots, totaling 10 replicates per blood sample per condition, and these disks were analyzed by the dual assay as above. A center point of 25.0 °C and 50% relative humidity was also run each day in a fourth chamber. The actual temperature and humidity achieved inside each chamber was logged and used for analysis.

#### STATISTICAL METHODS

All analysis was carried out in R version 2.15.1 *(15)*.

#### COMPARISON OF DBS AND SERUM

We converted DBS results to serum-equivalent concentrations by dividing by (1 − hematocrit). Serum and serum-equivalent DBS $\log_{10}$ concentrations were then compared on a scatter plot and Bland–Altman plots. For free hCG$\beta$ in DBS, serum intact hCG was proposed to be a potential explanatory variable as well as serum free hCG$\beta$. We converted concentrations of the serum or serum-equivalent DBSs of these markers to molar concentrations. For free hCG$\beta$, concentrations in nanograms per milliliter were multiplied by $10^{-6}$ and divided by the molecular weight, 22 200 Da *(16)*. For intact hCG, for which standards had been calibrated against the fourth international standard for chorionic gonadotropin (75/589) from the National Institute for Biological Standards and Control (Hertfordshire, UK), the concentrations in units per liter were multiplied by (70/650), then by $10^{-6}$ (as per standard ampoule contents *(17)*), and then divided by the molecular weight, 36 700 Da. A DBS free-hCG$\beta$ model was fitted to predict $\log_{10}$ serum-equivalent DBS free-hCG$\beta$ molar concentration with $\log_{10}$ serum free-hCG$\beta$ and $\log_{10}$ serum intact-hCG molar concentrations.

#### DRYING EXPERIMENT

We fitted second-order polynomial mixed models to the data with blood sample numbers as random effects *(18)*. For free hCG$\beta$, the fixed-effects parameters were $T + RH + T{:}RH + RH^2$, and for PAPP-A, $T + RH + T{:}RH + T^2 + RH^2$, where $T$ is temperature and $RH$ is relative humidity and a colon indicates an interaction term. We chose 25 °C and 50% relative humidity as a reference condition and drew contour plots to show the percentage change from the reference condition concentration over the range of temperatures and relative humidities.

## Results

The maternal and screening demographic characteristics of the patient group are outlined in Table 1.

**Table 1. Demographic data.[a]**

| | |
|---|---|
| Ethnicity | |
| Asian | 132 (52.4) |
| White | 79 (31.3) |
| Black | 36 (14.3) |
| Other | 5 (2.0) |
| Smoking status | |
| Smoker | 23 (9.1) |
| Non-smoker | 229 (90.9) |
| Maternal weight, kg | 65.0 (56.0–74.0) |
| Maternal age, years | 29.4 (26.0–32.3) |
| Gestational age, days | 89.2 (86.5–89.3) |
| Hemoglobin, g/dL | 12.4 (11.7–13.1) |

[a] Data are counts (%) or medians (interquartile ranges).

#### ASSAY PRECISION

Within-spot within-run CVs were 5.94%, 6.77%, and 6.46% for PAPP-A at 0.2, 1.7, and 2.9 U/L and 4.83%, 4.72%, and 4.87% for free hCG$\beta$ at 15.2, 111, and 181.3 ng/mL. Between-run CVs were 10.39%, 12.37%, and 13.05% for PAPP-A and 7.63%, 9.70%, and 9.63% for free hCG$\beta$ at the same concentrations. Between-spot within-run variation was 6.82% for PAPP-A and 6.10% for free hCG$\beta$. The total CV for PAPP-A was 14.1%, and for free hCG$\beta$, 10.8%.

#### COMPARISON OF DBS AND SERUM

$\text{Log}_{10}$ serum concentrations of PAPP-A (mU/L) and free hCG$\beta$ (ng/mL) were used to predict their respective $\log_{10}$ DBS serum-equivalent concentrations, shown in Fig. 1. $\text{Log}_{10}$ serum-equivalent PAPP-A and $\log_{10}$ serum PAPP-A concentrations correlated well ($r = 0.982$), and $\log_{10}$ serum PAPP-A concentration was a good predictor of $\log_{10}$ serum-equivalent PAPP-A concentration; however, the serum-equivalent PAPP-A results were a mean of 10.2% (95% CI 8.7%–11.7%) lower than the corresponding serum results across the range of values measured.

$\text{Log}_{10}$ serum-equivalent free-hCG$\beta$ and $\log_{10}$ serum free-hCG$\beta$ concentrations correlated less well ($r = 0.959$). $\text{Log}_{10}$ serum free-hCG$\beta$ concentration underpredicted the $\log_{10}$ serum-equivalent free-hCG$\beta$ concentration. The serum-equivalent free-hCG$\beta$ results were a mean of 82.9% (95% CI 78.4%–87.6%) higher than the corresponding serum results across the range of values measured, although this ratio decreased with increasing concentration.

In the intact-hCG purification experiment, at least 99% of the serum hCG was removed in the purification

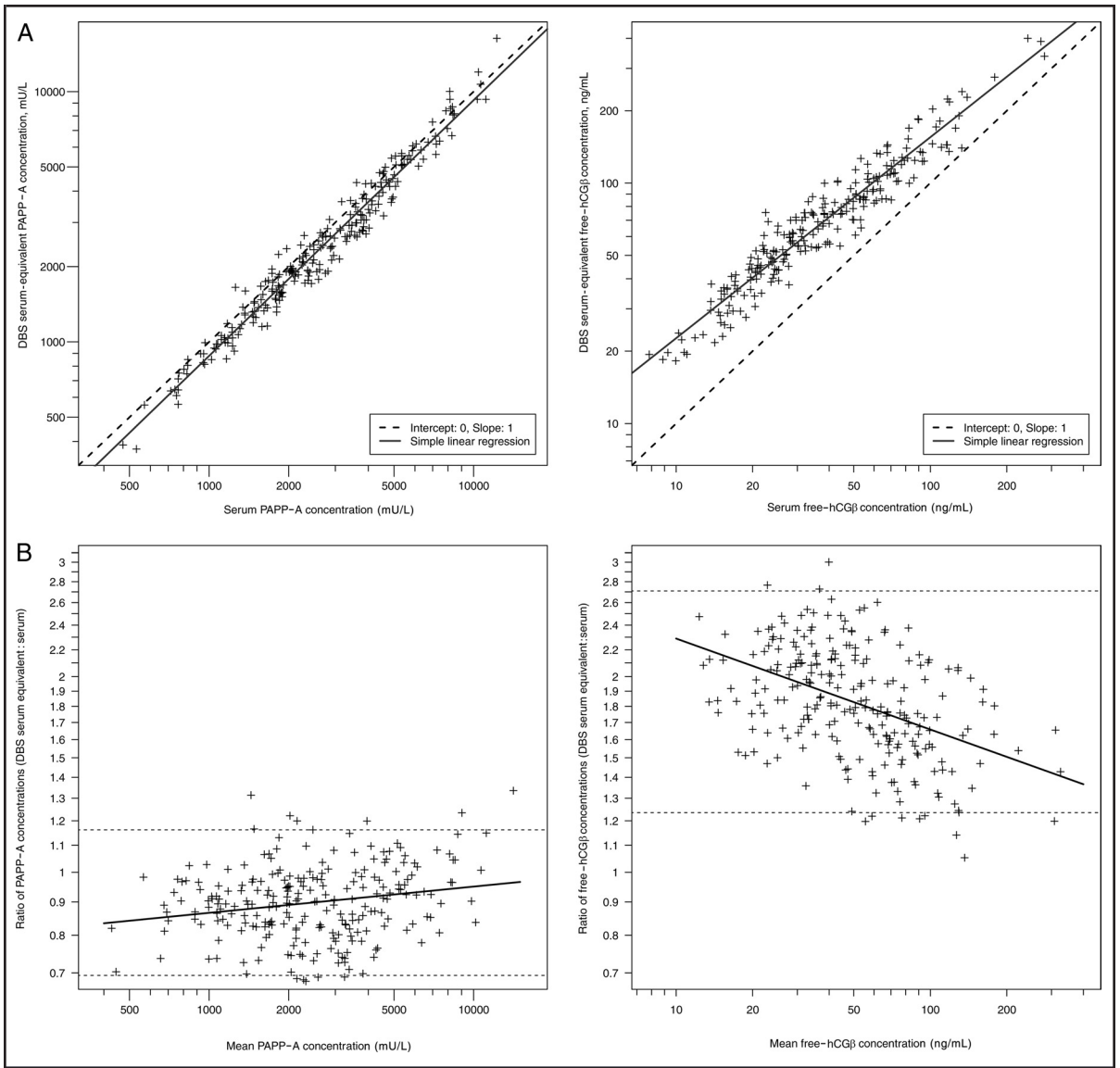


**Fig. 1. Comparisons between DBS and serum PAPP-A and free-hCG$\beta$ concentrations.**

(A), Scatter plots of PAPP-A and free-hCG$\beta$ serum and hematocrit-corrected DBS concentrations. A reference 1:1 line is shown as a dotted line, and the line of a simple least squares regression is shown as a solid black line. Left, $Log_{10}$ serum-equivalent PAPP-A concentration $= -0.12 + 1.02 \times \log_{10}$ serum PAPP-A concentration ($R^2 = 0.963$). Right, $Log_{10}$ serum-equivalent free-hCG$\beta$ concentration $= 0.52 + 0.84 \times \log_{10}$ serum free-hCG$\beta$ concentration ($R^2 = 0.920$). (B), Bland–Altman plots showing mean PAPP-A and free-hCG$\beta$ concentrations between the 2 methods on the *x* axis and the ratio of hematocrit-corrected DBS to serum concentrations on the *y* axis. Dotted lines represent limits of agreement (mean ratio + 1.96 SD of ratios); thick line is the slope between ratio and $\log_{10}$ mean values. Left, PAPP-A slope $= 0.040$, $P = 0.0013$. Right, Free-hCG$\beta$ slope $= -0.140$, $P < 0.0001$.

step. In nonpurified serum, the DBS:serum ratios were 110% and 80% for free hCG$\beta$ and PAPP-A, respectively. The corresponding ratios were 73% and 77% in purified serum. Purification thus decreased the free-hCG$\beta$ concentration by 33% but had only a negligible effect on the PAPP-A concentration, suggesting that the increased concentration of free hCG$\beta$ in DBS samples may be due to a release of extra $\beta$-subunits from the dissociation of intact hCG. Serum intact hCG was therefore also measured in the main study, and after

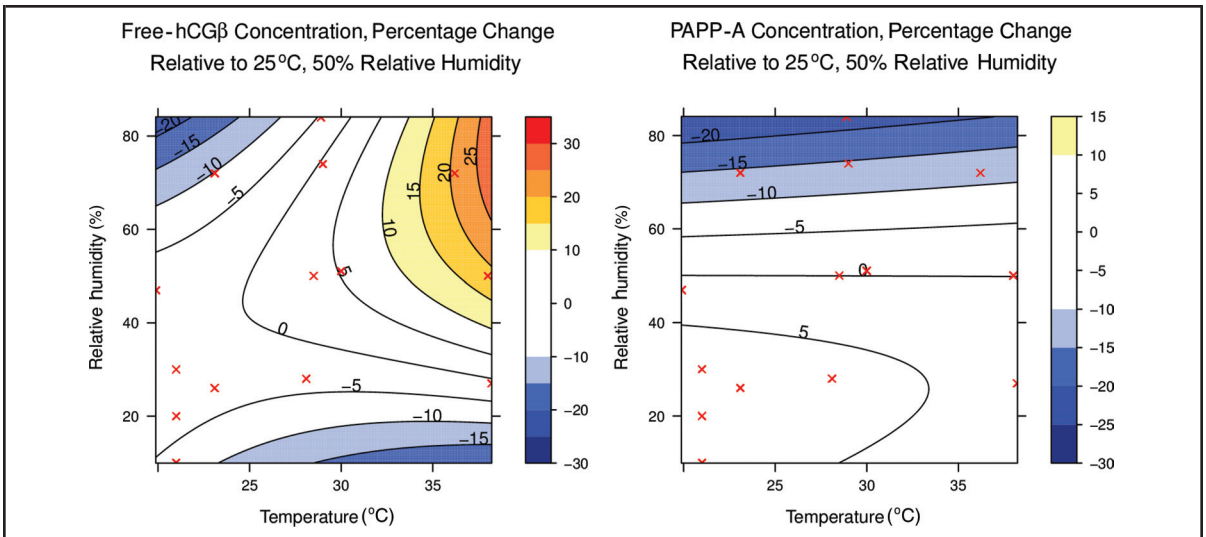


**Fig. 2. Contour plots showing the change in concentration (relative to 25 °C and 50% relative humidity) of free hCG$\beta$ and PAPP-A at different drying temperatures and relative humidities.**

Red crosses mark the combinations of temperature and relative humidity where measurements were made. A region where the concentration does not change beyond ±10% of the reference condition is shown in white, increases in measured concentration in yellow and orange shades, and decreases in blue shades.

conversion to molar concentrations, the following model was fitted:

$$\log_{10}\text{ serum-equivalent free-hCG}\beta\text{ molar concentration} = -0.53 + 0.57 \times \log_{10}\text{ serum free-hCG}\beta\text{ molar concentration} + 0.44 \times \log_{10}\text{ serum intact-hCG molar concentration } (R^2 = 0.960).$$

#### DRYING EXPERIMENT

The effects of temperature and humidity during the blood spot drying process on the concentrations of PAPP-A and free hCG$\beta$ are shown in Fig. 2, relative to the reference condition of 25 °C and 50% relative humidity. With a risk cutoff of 1:200, it has been demonstrated that at worst a 10% negative bias in PAPP-A would increase the false-positive rate from 4.5% to 5.6%, and a 10% positive bias would decrease the detection rate from 89% to 87%. With free hCG$\beta$, a 10% positive bias would increase the false-positive rate from 4.5% to 5.3%, and a 10% negative bias would decrease the detection rate from 89% to 88% *(9)*. A region of ±10% change of concentration relative to the reference condition therefore appears shaded white in the figure, representing an acceptable and manageable deviation in concentration of each marker. Increases in measured concentration are represented by shades of yellow and orange, and decreases by shades of blue. At high temperature and relative humidity, free-hCG$\beta$ measurements begin to increase, whereas at low temperatures and very high relative humidity they decrease. Temperature has little effect on the measured concentrations of PAPP-A, although at high relative humidity, PAPP-A measurements begin to decrease.

## Discussion

Hematocrit-corrected PAPP-A concentrations measured in DBS on the AutoDELFIA PAPP-A/Free hCG$\beta$ dual assay were comparable to those measured in serum from the same subject. However, hematocrit-corrected DBS free-hCG$\beta$ concentrations were higher than their serum counterparts. Artificial blood made from pregnant serum resulted in a similar overrecovery of free hCG$\beta$ in DBS; however, overrecovery did not occur in blood spots made of intact hCG–free serum. This suggested that the DBS assay was measuring a composite of true free hCG$\beta$, as measured in serum, plus additional $\beta$-subunits of hCG that dissociated from intact hCG during the blood spot drying process.

Inadequate sample handling can lead to an overestimation of serum free-hCG$\beta$ concentrations due to the release of $\beta$-subunits from intact hCG in the test tube. Free-hCG$\beta$ concentrations increased by 10% after 3 days when left in serum at room temperature, and after just 12 h when stored at 30 °C *(7, 8)*. Samples transported or stored as whole blood had even faster increases in free-

hCG$\beta$ concentration. In DBSs, once dried, free hCG$\beta$ was more stable than in serum *(13)*. PAPP-A, in contrast, was less stable in DBSs than in serum, although it changed at a slower rate than serum free hCG$\beta$.

In the present study, we found that individually, both marker concentrations were unaffected up to a $\pm$10% change in marker level, provided the spots were dried in conditions of $<$32 °C and 70% relative humidity. However, high temperature and high humidity caused a decrease in PAPP-A concentrations as well as an increase in free-hCG$\beta$ concentrations. An increase in free-hCG$\beta$ concentrations corresponded to the breakdown of intact hCG releasing extra $\beta$-subunits; therefore, high temperature and humidity resulted in decreased stability of PAPP-A and intact hCG. Presumably this was due to high temperatures increasing the rate of decay and high humidity slowing down the drying time and increasing time available for decay.

Particular attention must be paid to the scenario of increased humidity and temperature. Because this bias is in the direction of the marker profile for a trisomy 21 pregnancy, if left unaccounted for, it would result in inflated risks and a large increase in the screen-positive rate. In hot and humid countries, blood spot samples have to be dried in a climate-controlled environment. Prompt shipment to the analytical laboratory is also recommended owing to the potential instability of PAPP-A *(13)*.

The present study also showed that absolute free-hCG$\beta$ concentrations are significantly higher in DBSs, and there was significant nonproportionality, as shown in the Bland–Altman plots of Fig. 1. The addition to the model of intact-hCG serum concentration increased the $R^2$ to 0.96. It is therefore likely that the increased observed concentration of free hCG$\beta$ in the DBSs resulted from dissociation of some hCG$\beta$ from the intact molecule during the drying process.

Krantz et al. *(12)* have recently assessed the screening performance of the AutoDELFIA DBS dual assay in the first trimester. In serum screening, trisomy 21 pregnancies typically have a first-trimester free-hCG$\beta$ median MoM of 1.98 *(19)*. In DBS screening, Krantz et al. *(12)* report a less-discriminating trisomy 21 free-hCG$\beta$ median MoM of just 1.68. Despite this, they were able to report detection rates comparable to those of serum screening, which was ascribed to a narrower free-hCG$\beta$ $\log_{10}$ MoM SD, 0.194 in 1064 retrospectively analyzed unaffected controls, compared to a typical free-hCG$\beta$ $\log_{10}$ MoM SD of unaffected controls in serum screening of 0.254 *(2)* or 0.265 *(20)*.

$\text{Log}_{10}$ intact-hCG MoM has a tighter SD in unaffected controls of 0.195 compared to that of serum $\log_{10}$ free-hCG$\beta$ MoM, reported above *(20)*. By simulation using Serum Urine and Ultrasound Screening Study (SURUSS) population parameters for serum hCG and free hCG$\beta$ *(20)* (see the Data Supplement, which accompanies the online version of this article at http://www.clinchem.org/content/vol59/issue6), we have been achieving a SD of 0.22 at all gestational weeks for $\log_{10}$ free-hCG$\beta$ DBS MoM produced by our composite model, similar to the level reported by Krantz et al. *(12)*. The median MoM in affected pregnancies in our simulation is 1.60, similar to the median MoMs reported by Krantz et al. *(12)*.

Using the variance-covariance data from our simulation, we have shown that DBS screening can feasibly achieve detection rates similar to those attained in serum screening (see online Supplemental Table 1). At weeks 12 and 13, screening with DBS may even be able to accomplish slightly better detection rates than serum screening, probably owing to the lower DBS free-hCG$\beta$ SD and the contribution from intact hCG, the marker performance of which improves for trisomy 21 throughout the first trimester *(21–23)*. However, it is clear from these data that screening algorithms will need to incorporate specific DBS parameters for means, SDs, and correlations and that these will be significantly different from those embedded in current Fetal Medicine Foundation algorithms.

For both analytes, the between-run CVs in the DBS assay were high, indicating that there is additional error associated with the blood spotting process. Furthermore, the DBSs in this study were created with a venipuncture tube, potentially underestimating the variability of DBSs collected from finger pricks. In automated serum screening, between-run CVs are typically around 3% *(24, 25)*, whereas between-run CVs were around 9% for PAPP-A and 12% for free hCG$\beta$ in DBS assays in the present study. However, the positive influence of a more stable free hCG$\beta$ with a tighter MoM SD in DBS measuring a composite of free and intact hCG may outweigh the negative impact of poorer imprecision of this method.

Increased analytical variation can add considerably to the variation in overall risk for trisomy 21. Assays with between-day CVs of around 2%–3% can produce a risk with a variability of around 7%, which is doubled when the between-day CVs rise to around 6% and rises to 25% when CVs are around 9% *(26)*. However overall error in risk estimation has not been thoroughly investigated in the context of screening for trisomy 21, and there are many components to the screening program which themselves may add significant error, including analytical variability, sample stability/degradation, gestational dating errors, assay bias, kit lot changes, and between- and within-person biological variability. Although analytical variability is a known and easily estimated quantity, as is (for some analytes) assay bias, for many of these other variables the impacts are not known or are difficult to quantify.

Therefore defining a clinically allowable error *(27, 28)* or overall performance criteria (total allowable error) becomes more complex. One method that has been used extensively in general clinical chemistry is to relate total measurement variability to biological variability and analytical variability *(29)*. In the context of aneuploidy screening, there are very limited data on within-person within pregnancy biological variability. The variability for free hCG$\beta$ and PAPP-A in the first trimester is on the order of 21% and 32%, respectively, in 1 published study *(30)*, and data suggest this is related to time between repeat samplings; an estimate of between-day variability is probably much less, at around 5% *(31)*, making a total measurement variability of one-half biological variability of around 2.5%. However, in the context of screening, analytical performance far superior to biological variability may be irrelevant, if one sets aside the complexities of other errors such as those associated with sample stability and gestational dating errors. In effect, such assays that control the issues of sample stability despite high imprecision may still be fit for the context in which they are used. Although currently there are no external quality assessment (EQA) programs for blood spot aneuploidy screening assays, the practical issues of EQA have been well addressed in the area of neonatal blood spot programs *(32)*.

This study identified some differences between the measurement of free hCG$\beta$ in a traditional serum assay and a new DBS dual assay. Users need be aware that the DBS assay will measure extra free hCG$\beta$ in addition to that measured on a serum assay and that new median gestational age–related concentrations of both markers in unaffected pregnancies are required, as well as specific blood spot population parameters in the screening algorithms. In addition, careful attention must be taken with the drying and shipping of DBS samples to ensure that high-quality PAPP-A and free hCG$\beta$ results are reported.

**Author Contributions:** *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.*

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