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PAPP-A and free ß-hCG stability in first trimester serum using PerkinElmer AutoDELFIA® and DELFIA® Xpress systems

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Background In this study we aim to investigate the stability of free-β-hCG and PAPP-A over time in serum and whole blood in typical routine temperatures.

Methods Serum pools were stored under the following temperatures: 30 °C, room temperature, refrigerator temperature and −20 °C, for up to 240 days. Stability of the markers in whole blood was examined in a shorter study and compared to serum. Samples were analysed using the AutoDELFIA® and DELFIA® Xpress analysers.

Results On the AutoDELFIA®, considering a 10% change acceptable, PAPP-A levels are stable in serum for 142 days at refrigerator temperature, 37 days at room temperature and 20 days at 30 °C. Free-β hCG is stable in serum for 94 days at refrigerator temperature, 3 days at room temperature and 12 h at 30 °C. There was no significant change with either analyte after −20 °C storage for up to 240 days or after six repeated freeze–thaw cycles. In whole blood, free-β hCG levels increased more rapidly compared to serum, especially at 30 °C.

Conclusion Normal handling of samples is only likely to minimally effect the risk assessment of chromosomal anomalies. However, careful attention should be paid to minimise the increase of free-β hCG levels in samples shipped as whole blood. Copyright © 2009 John Wiley & Sons, Ltd.

KEY WORDS: PAPP-A; free-beta-hCG; screening; stability; first trimester

INTRODUCTION

First trimester maternal serum measurements of free-β human chorionic gonadotrophin (hCG) and pregnancy-associated plasma protein-A (PAPP-A) combined with maternal age and ultrasound fetal nuchal translucency (NT) measurements form the combined test for risk assessment of trisomy 21 (T21) and other chromosomal anomalies (Spencer *et al*., 1999). Not only does this method offer better detection rates than second trimester screening methods, but screening programs in earlier pregnancy are preferred by expectant mothers (Spencer and Aitken, 2004).

hCG is a heavily glycosylated pregnancy hormone consisting of distinct α- and β-subunits (Cole, 1997). The subunits non-covalently join to form intact hCG, or are released as free, non-biologically active subunits. Bioassays measure total hCG (intact hCG and free-β hCG) by using capture and detection antibodies which target epitopes on the β-subunit accessible in both forms. Free-β hCG bioassays target epitopes which are not detectable when bound to the α-subunit. The free-β hCG marker is favoured over total hCG in the detection of chromosomal anomalies because free-β hCG levels are increased in T21 cases in the first trimester, when total hCG levels are still normal (Spencer and Macri, 1992) and offer added detection in the second trimester (Spencer *et al*., 1992). In trisomy 13 (T13) and trisomy 18 (T18) pregnancies, free-β hCG are reduced (Spencer, 2007). hCG can dissociate into its subunits, therefore free-β hCG may increase throughout sample storage due to the instability of intact hCG.

PAPP-A is a large glycoprotein that belongs to the metzincin superfamily of zinc metalloproteinases. During pregnancy, PAPP-A is secreted from trophoblasts (Bonno *et al*., 1994) and is found in maternal serum bound to the proform of major basic protein (proMBP) in a heterotetrameric complex of two PAPP-A subunits disulfide bonded to two molecules of proMBP (Oxvig *et al*., 1993). PAPP-A levels are found to be decreased in T21, T13 and T18 pregnancies in the first trimester (Spencer, 2007).

In routine screening, samples are subjected to inconsistent temperature conditions. Samples are refrigerated or left at room temperature for varying times, and when sent via post or courier, samples can be subjected to the increased temperatures, especially in warmer countries. In research, serum samples are often frozen for long periods of time before analysis. To be confident in the reproducibility of laboratory result, awareness of any thermally induced dissociation effects or molecular

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conformational changes of biomarkers in samples is of major importance.

In this study we evaluated the stability of free-β-hCG and PAPP-A over time in serum and whole blood in various typical storage and shipment temperatures using AutoDELFIA® and DELFIA® Xpress analysers (PerkinElmer, Turku, Finland).

MATERIALS AND METHODS

Serum study

Maternal serum pools were created using excess serum from samples that were collected as part of a routine prenatal screening program at King George Hospital, Essex. Blood was drawn in Greiner Bio–One Vacuette 6-ml serum tubes (item number 456 089), allowed to clot for 15 min and then centrifuged. Serum was drawn off and analysed by the screening clinic, then left for up to 3 h at refrigerator temperature prior to pooling. For each temperature condition, three pools were created on separate days. In total 12 pools were created for the following temperature conditions: ~30 °C, room temperature (~22 °C), refrigerator temperature (~4 °C) and freezer temperature (~−20 °C). An additional three pools were collected to study the effect of repeat freeze–thaw. Each pool was between 5 and 9 ml, consisting of between 6 and 14 fresh serum samples. First trimester samples were used except when not enough was available, in which case one or two samples from early second trimester patients could be used. After collecting the pools were gently mixed and then divided into 600-µl aliquots in 5-ml polypropylene tubes. One aliquot was immediately measured for free-β hCG and PAPP-A levels to attain $t = 0$ values, and the remaining aliquots we placed at the appropriate storage temperature, temperature loggers were used to record the condition temperatures. Subsequent measurements were made using new aliquots at later time points, as per the study schedule (Table 1). It was anticipated that free-β hCG was going to be less stable than PAPP-A, based on previously published work, so in general, free-β hCG was tested more frequently for a shorter overall length of time than PAPP-A. Following analysis the aliquots were discarded. For the freeze/thaw study the aliquots to be tested were thawed at room temperature and frozen 1, 2, 4 or 6 times before the analysis. As soon as the aliquots had defrosted, they were re-frozen.

Whole blood study

Four × 5-ml blood samples (one for each time period) were drawn from nine pregnant volunteers (3 per temperature) in BD Vacutainer Serum Tubes, (item number 367 614; siliconised glass tubes with no additive). Samples were gently inverted, and left to clot for either 30 min, 5, 24 or 72 h and then spun. The serum was removed and placed into a polypropylene tube and frozen at −20 °C. The samples were stored for 5–22 days, thawed and analysed in two batches.

Table 1—Approximate study schedule. Due to different start days and the falling of weekends, runs were sometimes made at slightly altered times

free-β hCG serum study	PAPP-A serum study
Refrigerator temperature	**Refrigerator temperature**
$t = 0$, 1, 3, 6, 9, 14, 29, 38, 51 days	$t = 0$, 1, 3, 6, 9, 14, 29, 38, 51 days
Room temperature	**Room temperature**
$t = 0$, 1, 2, 3, 8, 9, 11, 14 days	$t = 0$, 1, 4, 5, 6, 7, 12, 14, 19, 20, 21, 25, 27, 32 days
30 °C	**30 °C**
$t = 0$, 4 h, 10 h, 1, 2, 3 days	$t = 0$, 4 h, 10 h, 1, 2, 3, 6, 8, 10, 17, 20, 23, 27, 30 days
−20 °C	**−20 °C**
$t = 0$, 7, 14, 29, 63, 91, 179, 210 days	$t = 0$, 7, 14, 30, 64, 92, 184, 240 days

Sample analysis

Aliquots were all measured in triplicate with quality controls on the AutoDELFIA® platform, a solid phase two site fluoroimmunometric assay based on direct sandwich technique. Two monoclonal antibodies, derived from mice, are directed against two distinct antigenic determinants on the free-β hCG or PAPP-A/proMBP complex, respectively in a fully automated process. free-β hCG assay : free-β hCG molecules in samples, controls and calibrators react with the immobilised capture antibody and the detection samarium-labelled antibody. Following 2.5 h incubation and a washing step, DELFIA® Enhancement solution dissociates the samarium ions which form highly fluorescent chelates with components of the Enhancement solution. PAPP-A assay : an additional first step is required to immobilise the biotinylated capture assay to the streptavidin coating the well walls. Following a washing step, PAPP-A molecules in samples, controls and calibrators react with the immobilised capture antibody and the detection europium-labelled antibody. Following 2 h incubation and a washing step, DELFIA® Enhancement solution dissociates the europium ions which form highly fluorescent chelates as in the free-β hCG assay. In both assays the fluorescence is measured and the result is proportional to the concentration of analyte in the sample.

Room temperature and freeze–thaw serum samples and all whole-blood samples were additionally measured for free-β hCG and PAPP-A on the DELFIA® Xpress random access system for comparison. Briefly, DELFIA® Xpress uses cups which are essentially a single well of a 96-well plate and each cup is treated individually. Calibration occurs once per kit lot, and QCs are run before a batch of samples. The chemistry is the same as the AutoDELFIA® except that in both assays the capture antibody is prebound to the solid wall, europium-labelled detection antibodies are used and DELFIA® Inducer is used in place of DELFIA® Enhancement solution. DELFIA® Xpress incubation is also a lot shorter at 20 min, and reports results 30 min after starting a run.

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The analysis centre has ethics approval in order to use the remaining excess serum for research purposes.

Statistics

Mean marker values were calculated from the triplicate results for each time point for each storage temperature. Results were plotted at each time point as percentage of concentration at $t = 0$ and analysed by linear regression.

RESULTS

Figures 1 and 2 show stability plots for free-β hCG and PAPP-A at refrigerator temperature, room temperature, 30 °C and −20 °C measured on the AutoDELFIA®.

Using the linear regression lines shown in Figures 1 and 2, the number of days before each analyte is likely to have a 10% change from $t = 0$ as measured by the AutoDELFIA® was 142 for PAPP-A and 94 for free-β hCG at refrigerator temperature. At room temperature (22 °C) this was 37 days and 3 days respectively, and at 30 °C, this was 20 days and 12 h. At room temperature, the results from the DELFIA Xpress® confirmed the findings for free-β hCG stability, but deviated from the findings for PAPP-A, finding it to be stable for only 16 days at room temperature on this analyser, rather than 37 on AutoDELFIA®.

For −20 °C there is no significant concentration change during the 210 days (free-β hCG) or 240 days (PAPP-A) studied.

Table 2 shows the results from the whole blood stability study on the AutoDELFIA® compared to serum stability determined using regression lines in Figures 1 and 2. The whole blood study results for the DELFIA Xpress® were comparable to those of the AutoDELFIA®.

Two to six freeze–thaw cycles had no effect on the levels of either PAPP-A or free-β hCG, shown in Table 3.

Temperature loggers recorded mean temperatures (and range) as: 'refrigerator temperature': 3.0 °C (−1.5–8.0 °C), 'room temperature': 21.6 °C (20.5–23.0 °C), '30 °C': 28.9 °C (28.0–29.5 °C) and '−20 °C': −22.2 °C (−11.0–−27.0 °C).

DISCUSSION

We have shown that PAPP-A levels are stable (hereafter defined as <10% change from $t = 0$ (Anderson and Scott, 1991) in serum for 142 days at refrigerator temperature, 37 days at room temperature and 20 days at 30 °C, none of which pose a problem in any likely

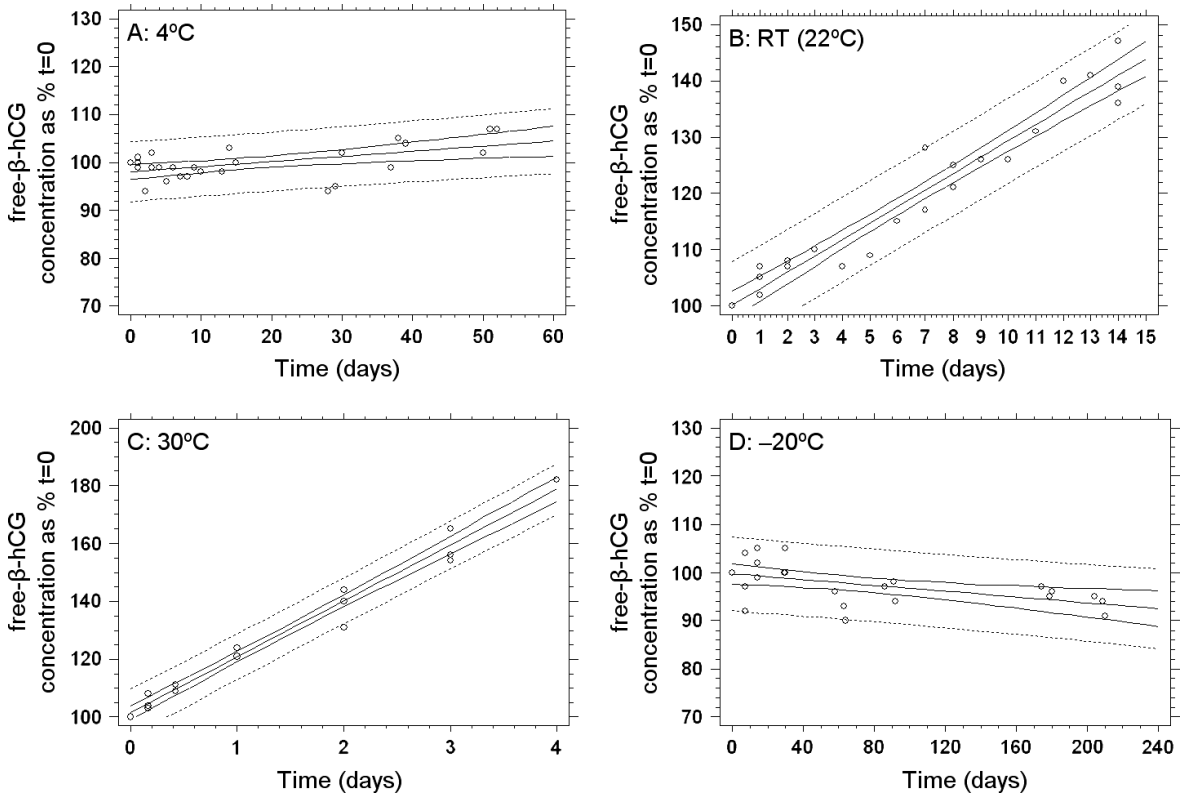

Figure 1—Serum sample free-β hCG stability results. The mean concentration of triplicates measured with the AutoDELFIA® Free hCGß kit compared to the result at 0-point. The outer limits (dotted lines) define the 95% prediction interval for new observations and the inner limits (filled lines) correspond to the 95% confidence interval for the fitted linear regression (middle filled line). (A) Refrigerator temperature. Fitted regression line y = 98.1 + 0.107× (B) Room temperature. Fitted regression line y = 100.1 + 2.920× (C) 30 °C. Fitted regression line y = 101.5 + 19.311× (D) −20 °C. Fitted regression line y = 99.7–0.030×

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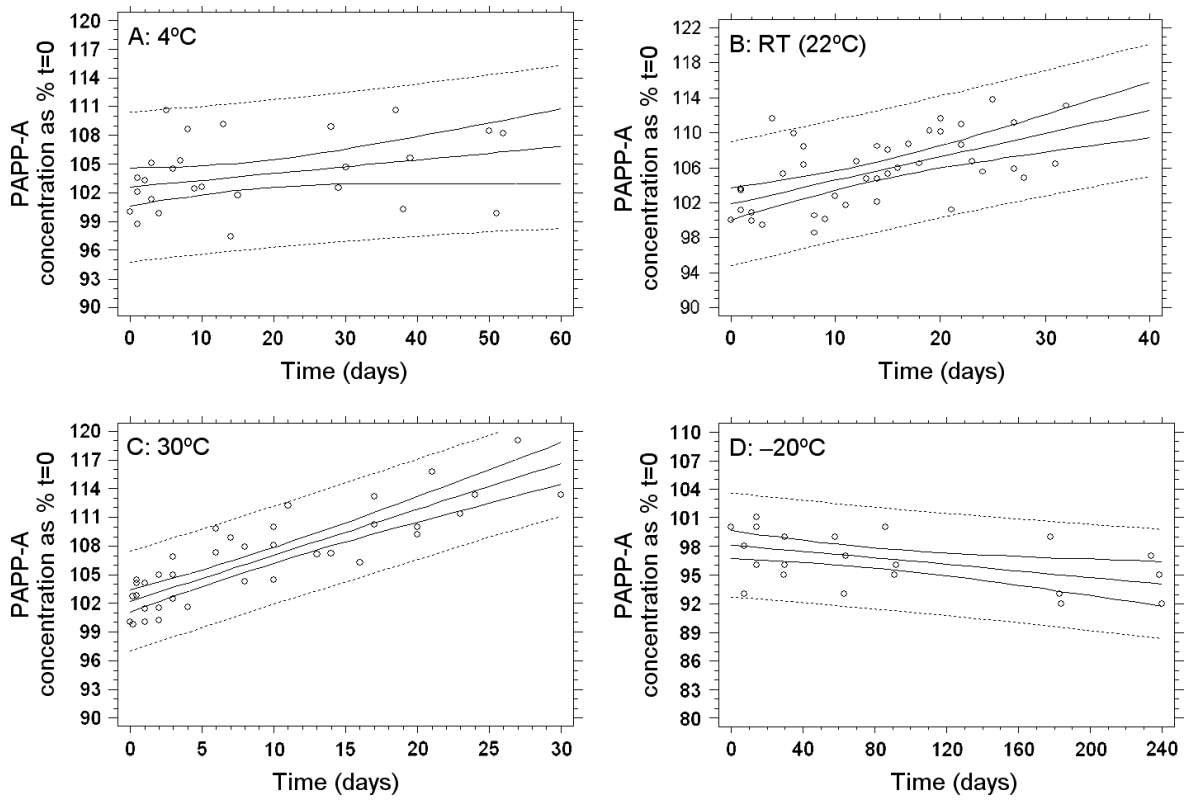

Figure 2—Serum sample PAPP-A stability results. The mean concentration of triplicates measured with the AutoDELFIA® PAPP-A kit compared to the result at 0-point. The outer limits (dotted lines) define the 95% prediction interval for new observations and the inner limits (filled lines) correspond to the 95% confidence interval for the fitted linear regression (middle filled line). (A) Refrigerator temperature. Fitted regression line $y = 102.6 + 0.070\times$ (B) Room temperature. Fitted regression line $y = 101.9 + 0.269\times$ (C) 30 °C. Fitted regression line of $y = 102.2 + 0.481\times$ (D) −20 °C. Fitted regression line $y = 98.2 - 0.017\times$

Table 2—Stability of PAPP-A and free-β hCG in whole blood and comparison in serum measured with the AutoDELFIA®. Serum levels were calculated from the regression lines in Figures 1 and 2

	PAPP-A average % recovery		Free-β hCG average % recovery	
Hours	Whole blood	Serum	Whole blood	Serum
	Refrigerator temperature			
5	97.1	102.6	99.5	98.1
24	95.9	102.7	98.2	98.2
72	103.4	102.8	100.9	98.4
	Room temperature			
5	99.2	102.0	101.7	100.7
24	102.7	102.2	104.7	103.0
72	106.3	102.7	110.1	108.9
	30 °C			
5	100.5	102.3	110.2	105.5
24	104.6	102.7	140.8	120.8
72	104.0	103.6	365.8	159.4

screening setup. We have also shown PAPP-A to be stable for at least 3 days in whole blood. The suitable stability of PAPP-A in screening has been shown before (Bersinger *et al*., 1995; Qin *et al*., 2002; Lambert-Messerlian *et al*., 2006).

Table 3—−20 °C to room temperature freeze–thaw cycle stability of free-β hCG and PAPP-A in serum measured with the AutoDELFIA®

Number of freeze–thaw cycles	Average recovery compared to reference (%)	
	Free-β hCG	PAPP-A
1 (reference)	100.0	100.0
2	99.3	101.8
4	99.2	101.3
6	98.9	101.1

Following the introduction of free-β hCG as a marker in chromosomal anomaly screening (Macri *et al*., 1990; Spencer, 1991), and an anecdotal claim of possible room temperature and ambient shipping temperature instability of this marker (Knight and Cole, 1991), several studies have investigated the stability of free-β hCG. Intact hCG is a dissociable dimer producing free α- and β-subunits. free-β hCG is a minor component of normal pregnancy serum hCG (<1% of the total hCG concentration), therefore, even small amounts of dissociation of intact hCG can potentially swamp free-β hCG levels (Cole, 1997).

In the present study, we have shown that at refrigerator temperature, room temperature and 30 °C there is an apparent increase in free-β hCG in serum over time

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when measured on the AutoDELFIA®, due to intact hCG releasing new free-β subunits. The rate of free-βhCG increase is temperature dependent. At room temperature and 30 °C, free-β hCG is stable in serum for only 3 days and 12 h respectively. In whole blood, we have found the rate of hCG dissociation to be faster than in serum, especially at 30 °C, shown in Table 2.

It has previously been shown that free-β hCG levels increase in whole blood over time, being stable for 34 h at 22 degrees (Spencer *et al*., 1993). Similar increases were found by others studying whole blood at room temperature: 10% free-β hCG increase in 24 h (Wald *et al*., 1993), 14% increase in 24 h (Stevenson *et al*., 1993), 9.9% increase in 24 h (Sancken *et al*., 1995), 10.2% increase in 24 h (Beaman *et al*., 1996), average 14% increase per day over 4 days (Stone and Henley, 1995). However, by examining the median MoMs of samples which had been in the postal system for different numbers of days, the actual increase in free-β hCG concentration at longer transit times has been shown to be much less than this, 3.4–3.8% for 4 days (Cuckle and Jones, 1994, 1995), possibly due to lower than room temperature levels during transportation in the United Kingdom. The change was found to be higher in the summer months, which was confirmed in France: over 4 to 8 days in the post, a 3.0% increase in median level was found in winter versus 13.0% increase in summer (Muller *et al*., 1999). Both of these groups concluded that instability did not significantly reduce the quality of free-β hCG results in routine care, especially if samples were separated into serum rapidly after collection and that transit time was minimised.

Dissociation of intact hCG to release free-β hCG has also been previously shown to be slower in separated blood, increasing by just 3% in serum after 24 h at room temperature (Zimmermann *et al*., 1994) and being stable for 87.3 h at 20 °C in serum (Spencer *et al*., 1993) and 90 h in plasma at room temperature (Beaman *et al*., 1996).

In this study we have shown that when stored at refrigerator temperature, free-β hCG is considerably more stable, remaining with less than a 10% change for 94 days. An increased stability at refrigerator temperature compared to room temperature has also been previously found. free-β hCG has been shown to be stable for 96 h in whole blood (Beaman *et al*., 1996), or to have no significant change after 72 h in whole blood (Sancken *et al*., 1995), and in serum to be stable for 115 days at refrigerator temperature (Spencer *et al*., 1993).

Using the data from one report (Spencer *et al*., 1993), we recalculated the regression line for their Arrhenius plot and from this predicted the stability of free-β hCG in serum based on this at 22 °C and 30 °C to compare to the results from our study. The predictions are remarkably close to the results from this study, shown in Table 4.

Plasma samples have been shown to display a small but significant decrease in free-β hCG concentrations after 6 months storage at −20 °C (Beaman *et al*., 1996), similar to the downward trend we found in serum at this temperature, (Figure 1D), suggesting a possible very slight denaturing effect on free-β hCG caused by long term frozen storage. The same group found storage of plasma at −70 °C had no effect on stability (Beaman *et al*., 1996). In the present study, it has been found that the freeze-thawing process has a minimal effect on stability, as previously reported (Spencer *et al*., 1993; Wald *et al*., 1993; Spencer and Macri, 1994; Masse *et al*., 1997).

Table 4—Stability of free-β hCG in serum predicted from Arrhenius equation in Spencer *et al*. (1993)

Test temperature (°C)	Predicted stability	Results from present study
−20	131 years	Not determined
4	115 days	94 days
22	2.4 days[a,b]	3 days
30	12 h[a]	12 h

[a] Predicted after recalculating Arrhenius plot regression line from original table data.
[b] Experimental finding within study (Spencer *et al*., 1993) showed free-β hCG to be stable for 2.9 days in serum at 22 °C.

Cleavage of a single peptide link at one of three sites on the beta subunit of hCG, known as 'nicking', decreases stability of intact hCG thereby speeding up the dissociation of the dimer (Kardana and Cole, 1997). It was found that addition of penicillin-streptomycin-amphotericin concentrate dramatically slowed down the increase in detection of free-β hCG in serum stored at 21 degrees over 4 weeks, suggesting that microbial enzymes were responsible for the nicking and subsequent dissociation of intact hCG (Kardana and Cole, 1997). Other attempts to investigate stabilising hCG include the use of protease inhibitors. Although sodium azide has no effect (Spencer *et al*., 1993), sodium idoacetate, a cysteine protease inhibitor, effectively stopped dissociation at room temperature (Stevenson *et al*., 1993), although it had no effect at 30 °C (Sancken *et al*., 1995). In addition, improved stability has been shown using dried blood spots (Spencer *et al*., 1993; Perni *et al*., 2006).

In conclusion, the effects of normal handling of samples is at most only likely to minimally effect the risk assessment of chromosomal anomalies on the AutoDELFIA® and DELFIA® Xpress platforms. However, laboratories measuring free-β hCG for screening should continue to take notice of sample handling prior to analysis, since long exposure to room temperatures or higher could increase results due to the release of the free-β subunit from intact hCG dissociation. Ensuring serum samples are stored at refrigerator temperature prior to analysis effectively removes this issue. In practice, this is not always possible where samples are transported centrally from large catchment areas where post at ambient temperatures is the only affordable option. It is probably not feasible to introduce a correction factor for transported samples for free-β hCG testing due to the large variance in transport times and temperatures; however, if not already established, laboratories should issue a standard operating procedure for sample handling and receipt in this regard. After our original stability results in 1993 (Spencer *et al*., 1993), we introduced the following instruction to reception staff:

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Whole blood samples received in excess of 48 hours or serum samples received in excess of 72 hours must be rejected and reported with the comment "Whole-Blood Sample delayed in transit in excess of 48 hours/Serum sample delayed in transit in excess of 72 hours—this is likely to have resulted in deterioration of the sample and will produce an inaccurate risk estimate. Please re-bleed the patient and send to the laboratory within a maximum of 48 hours/72 hours."

This process has enabled us to provide effective screening programs in the first and second trimester over the past 16 years (Spencer, 1999; Nicolaides *et al*., 2005).

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