

measurement associated with patients' results (5).

#### References

1. Fraser CG, Harris EK. Generation and application of data on biological variation in clinical chemistry. *Crit Rev Clin Lab Sci* 1989;27:409–37.
2. Bokelund H, Winkel P, Statland BE. Factors contributing to intra-individual variation of serum constituents: 3. Use of randomized duplicate serum specimens to evaluate sources of analytical error. *Clin Chem* 1974;20:1507–12.
3. Fuentes-Arderiu X, González-Alba JM, Baltuille-Peirón F, Navarro-Moreno MA. Premetrological variation of some thyrotropin, thyroxine (non-protein bound), and triiodothyronine concentrations in serum. *Clin Chem* 2000;46:431–2.
4. Fuentes-Arderiu X, Acebes-Frieyro G, Gavasos-Navarro L, Castiñeiras-Lacambra MJ. Pre-metrological (pre-analytical) variation of some biochemical quantities. *Clin Chem Lab Med* 1999; 37:987–9.
5. Fuentes-Arderiu X. Uncertainty of measurement in clinical laboratory sciences. *Clin Chem* 2000; 46:1437–8.

Núria Monge-Azemar  
Xavier Fuentes-Arderiu\*

Servei de Bioquímica Clínica  
IDIBELL–Hospital Universitari de  
Bellvitge  
L'Hospitalet de Llobregat  
Catalonia, Spain

\*Address correspondence to this author at: Servei de Bioquímica Clínica, Hospital Universitari de Bellvitge, Catalonia, Spain. Fax 33-93-260-75-46, e-mail xfa@csub.scs.es.

DOI: 10.1373/clinchem.2004.039438

#### Stability of Whole Blood at $-70^{\circ}\text{C}$ for Measurement of Hemoglobin $\text{A}_{1\text{c}}$ in Healthy Individuals

To the Editor:

To our knowledge, there are no significant published data on the long-term stability of stored whole blood for hemoglobin  $\text{A}_{1\text{c}}$  ( $\text{HbA}_{1\text{c}}$ ) analysis. Stored hemolysates (1) and buffy coat samples (2) can provide reliable estimates of  $\text{HbA}_1$  and  $\text{HbA}_{1\text{c}}$ , respectively; in large epidemiologic studies, however, storage of whole blood would be more practical. A recent report stated that whole blood was stable at  $-70^{\circ}\text{C}$  for 1 year, but no evidence was cited (3). The aim of

this study was therefore to test the hypothesis that whole blood is stable at  $-70^{\circ}\text{C}$  for 1 year for  $\text{HbA}_{1\text{c}}$  measurement.

Venous blood was collected from 99 children (age, 10 years) without diabetes over a 3-week period in the Avon Longitudinal Study of Parents and Children (ALSPAC; www.alspac.bris.ac.uk). The anticoagulant used was lithium heparin. Available blood was limited, and 10 identical 5- $\mu\text{L}$  aliquots of whole blood in 1 mL of 40H-HPLC-hemolysant (specific to our assay; contains phosphate buffer, sodium azide, and surfactant) were prepared for each child. One aliquot was assayed fresh for  $\text{HbA}_{1\text{c}}$  (baseline values), and the remainder were frozen at  $-70^{\circ}\text{C}$ . Whole blood was also stored undiluted in hemolysant. Stored aliquots were assayed for  $\text{HbA}_{1\text{c}}$  after periods of 9, 16, 25, 31, 45, and 56 weeks. After 56 weeks, the stored undiluted whole blood from each child was also assayed.  $\text{HbA}_{1\text{c}}$  was measured with the HA-8140 Hi-Auto  $\text{HbA}_{1\text{c}}$  analyzer (Menarini Diagnostics) maintained in alignment with the Diabetes Control and Complications Trial method. Imprecision (CV) was stable at 1.8% (mean  $\text{HbA}_{1\text{c}}$ , 5.5%) throughout the study.

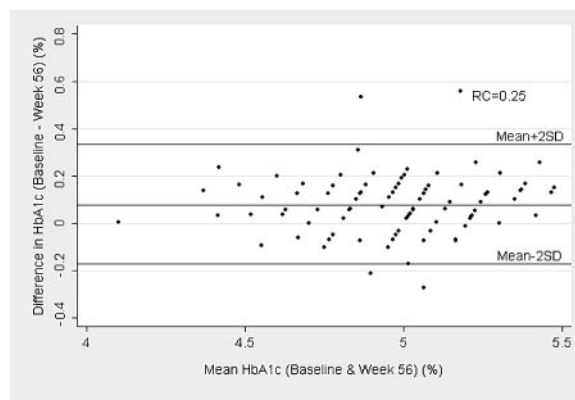
Baseline  $\text{HbA}_{1\text{c}}$  values were adjusted for day of blood collection (days 1 to 24; D) to allow similar storage periods to be compared for each child, using the following equation: adjusted  $\text{HbA}_{1\text{c}} = \text{HbA}_{1\text{c}} - \beta(\text{D} - \text{median D})$ , where  $\beta$  was derived from a regression of  $\text{HbA}_{1\text{c}}$  on D (adjustment only marginally altered values).  $\text{HbA}_{1\text{c}}$  distribution

was approximately gaussian. Trends in  $\text{HbA}_{1\text{c}}$  with storage (in samples diluted in hemolysant before freezing) were explored by linear regression using  $F$ -tests to determine the suitability of using a common slope and intercept for all individuals. The difference between  $\text{HbA}_{1\text{c}}$  at baseline and after 56 weeks of storage undiluted in hemolysant was assessed by  $t$ -test. Agreement between  $\text{HbA}_{1\text{c}}$  in fresh and stored blood was assessed by Bland–Altman analysis at each time point (4). Repeatability coefficients were calculated as twice the SD of the difference between  $\text{HbA}_{1\text{c}}$  in fresh and stored samples.

Mean (SD)  $\text{HbA}_{1\text{c}}$  was 5.0 (0.3)% at baseline and 4.9 (0.3)% after 56 weeks at  $-70^{\circ}\text{C}$ , in samples stored both diluted and undiluted in hemolysant. A common slope ( $P = 0.99$ ) with separate intercepts for each individual ( $P < 0.001$ ) represented the relationship between  $\text{HbA}_{1\text{c}}$  and storage period, and  $\text{HbA}_{1\text{c}}$  decreased by 0.0016% (95% confidence interval,  $-0.0021\%$  to  $-0.0012\%$ ) per week of storage, equating to  $-0.1\%$   $\text{HbA}_{1\text{c}}$  after 56 weeks. Mean  $\text{HbA}_{1\text{c}}$  was also lower, vs baseline, after 56 weeks of storage undiluted in hemolysant ( $P < 0.001$ ). Agreement between baseline and stored values was similar at each time point (for an example, see Fig 1). Differences between fresh and frozen measurements were unrelated to  $\text{HbA}_{1\text{c}}$  value; mean differences ranged from  $-0.001\%$  to 0.17%  $\text{HbA}_{1\text{c}}$ ; repeatability coefficients ranged from 0.23% to 0.28%; and limits of agreement were between  $-0.2\%$  and 0.4%. Variability could largely be explained by assay

Fig. 1. Agreement between  $\text{HbA}_{1\text{c}}$  measured in fresh whole blood (baseline) and in identical aliquots of whole blood stored at  $-70^{\circ}\text{C}$  for 56 weeks, without previous dilution in 40H-HPLC-hemolysant ( $n = 99$ ).

Baseline blood collection took place over a 3-week period, and baseline  $\text{HbA}_{1\text{c}}$  measurements have been adjusted for time. RC, repeatability coefficient.



imprecision, although it is impossible to exclude a slight downward assay drift of 0.1% over the 56-week study period.

In conclusion, whole blood stored at  $-70^{\circ}\text{C}$  for 1 year is stable for later measurement of  $\text{HbA}_{1\text{c}}$  by HPLC in individuals without diabetes. This eliminates the need to analyze fresh blood in epidemiology and potentially could permit  $\text{HbA}_{1\text{c}}$  to be measured in existing cohorts with stored whole blood.

#### References

1. Simon M, Hoover JD. Effect of sample instability on glycohemoglobin ( $\text{HbA}_{1\text{c}}$ ) measured by cation-exchange chromatography. *Clin Chem* 1982;28:195–8.
2. Youngman LD, Clark S, Manley S, Peto R, Collins R. Reliable measurement of glycated hemoglobin in frozen blood samples: implications for epidemiologic studies [Letter]. *Clin Chem* 2002;48:1627–9.
3. Sacks DB, Bruns DE, Goldstein DE, Maclaren NK, McDonald JM, Parrott M. Guidelines and recommendations for laboratory analysis in the diagnosis and management of diabetes mellitus. *Clin Chem* 2002;48:436–72.
4. Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet* 1986;1:307–10.

Wendy Jones<sup>1\*</sup>

Jeff Scott<sup>2</sup>

Sam Leary<sup>3</sup>

Fay Stratton<sup>3</sup>

Susan Smith<sup>3</sup>

Richard Jones<sup>3</sup>

Andrew Day<sup>2</sup>

Andrew Ness<sup>3</sup>

ALSPAC Study Team<sup>3</sup>

<sup>1</sup> Department of Social Medicine  
and

<sup>3</sup> Avon Longitudinal Study of Parents  
and Children  
University of Bristol  
Bristol, UK

<sup>2</sup> Department of Clinical Biochemistry  
United Bristol Healthcare NHS Trust  
Bristol, UK

\*Address correspondence to this author at: Department of Social Medicine, University of Bristol, Bristol, UK BS8 2PR. E-mail W.A.Jones@bristol.ac.uk.

DOI: 10.1373/clinchem.2004.038521

#### Additional Data for Oligonucleotide Arrays of the *p53* Gene in DNA from Formalin-Fixed, Paraffin-Embedded Tissue

To the Editor:

The report published by Cooper et al. (1) motivated us to report our experience with the Affymetrix<sup>®</sup> GeneChip<sup>™</sup> (Chip). In its current state, we agree that the Chip cannot be used as a stand-alone test for mutational analysis of the *p53* gene. However, it is still a sensitive tool for reducing the need for full-length sequencing. Validation studies in our laboratory have demonstrated that the Chip can detect most mutations in as few as 5% of a cell population; other claims run to as low as 2%.

The limitations of the Chip include a failure to detect insertions, large deletions, and  $>6$  bp of intronic sequence in any direction. Some limitations are attributable to sequence tiling (2). Insertions and large deletions were not tiled at all. As for the intronic sequences, only the splicing junctions were considered to be critical, thus limiting the utility of the Chip.

Failure to detect some missense mutations appears to be attributable to the quality of the DNA and the number of times a sequence has been tiled on the Chip. As noted by the authors (1), DNA recovery from paraffin-embedded tissue (PET) is dependent on many variables, including the age of the block, fixation, and isolation conditions. The quantity of tumor tissue present in the section can impact the ability to detect a mutation, particularly if the tissue is predominantly healthy, nondiseased stroma. Staining of tissue samples before isolation introduces inhibitors of the amplification process. To optimize detection, we suggest manual microdissection using a stained slide as a guide for dissecting the unstained slides. This process improves the ratio of tumor tissue in the isolation; a minimum of 5–10  $\mu\text{m}$  of unstained sections should provide sufficient DNA for analysis.

The focus of the report, however, was to describe the performance of

the Chip on PET samples. Our experience has been that poor performance for exon 4 and the lack of polymorphism detection can be explained predominantly by issues concerning DNA recovery from PET. In many cases, the Chip could not interrogate exon 4 because of amplification failure ( $\sim 32\%$ ). This amplification product is the largest (366 bp) in the multiplex, and given the compromised template, poor results are not unexpected. Alternative amplification conditions and/or exon-specific amplification can be used to overcome sample quality issues.

The investigators (1) also noted that the Chip lacks the ability to detect polymorphisms in PET. From a sampling of data collected over the past 2 years in our laboratory, 62 cancer samples were identified as originating from PET. Of the 62 samples, 34% (21 cases) were identified with a polymorphism in exon 4, 6, 7, or 8. The scoring assigned to these polymorphisms was consistently a "5" or a "6" and was confirmed by sequencing in most instances. The Chip identified one of six R72P polymorphisms in PET.

Lastly, we would like to address the application of a general algorithm for scoring cutoffs. Our primary cutoffs are set at 15 for the determination of mutation status; these are based on validation studies against sequencing, the "gold standard". Of the 62 samples that we analyzed using both Chip and sequencing, 37 mutations (missense, nonsense, or splice site) were detected by Chip, and 23 of these were detected by both methods. Six samples were determined to be wild type by both methods. Additionally, indications of other mutations were present in 16 samples. Of these, 44% (7) contained signals that were above the local background but were below the general cutoff scores of both methods. Our group and others have produced data that support the use of specific interpretive scoring of Chip positions to maximize interpretive accuracy.

In conclusion, we believe that the limitations noted by Cooper et al. (1) are related to interpretive as well as