ments *(14)*, it is possible that apparent increases in PTH are attributable to fragment detection, i.e., fragments unique to the degradation process in EDTA plasma; no such increase is observed in serum samples. Reassuringly, the increase is modest and of questionable clinical consequence.

The ideal specimen type remains unclear, but standardization of preanalytical handling is critical in clinical and research settings. If analysis occurs within 3 h, SST serum affords the most consistent results, regardless of acquisition and storage temperature. However, if a specimen remains at room temperature for several days before analysis, then EDTA-plasma results are less discrepant from their baseline. DPC does not make any recommendation to immediately cool SST specimens *(15)*. Our data support this recommendation and further suggest that a 3-h delay at room temperature will not affect results. When analysis must be delayed beyond 3 h, it is reassuring that even after 24 h, the mean decrease in SSR_{RT} results was only 7.3% (95% confidence interval, 3.2–11.2%), a change that failed to reach significance. DPC recommends that EDTA specimens be collected and maintained at 2–8 °C *(13)*. Our data suggest that this practice will prevent a paradoxical increase in PTH results, although we cannot comment on the necessity to precool EDTA tubes. In all cases, refrigeration minimizes effects of storage. We use room temperature SST serum for in-house tests and have verified and adopted the manufacturer's reference interval of 1.3–6.8 pmol/L. Referred-in samples are cooled or frozen as appropriate.

In summary, we examined samples from 31 hemodialysis patients, using two different matrices while varying acquisition temperature, storage temperature, and time to analysis. In the dialysis-dependent CKD population, EDTA-plasma specimens gave higher mean PTH results than SST serum. Mean PTH values from EDTA-plasma specimens collected and stored at room temperature increased modestly over time for the first 48 h. However, larger absolute percentage changes from baseline were seen in SST specimens similarly handled. Different reference intervals are needed for EDTA plasma and SST serum. The reference interval for room temperature plasma specimens should not be assumed to be identical to that for refrigerated plasma. If analyzed within 3 h of collection, SST specimens do not require cooling. As each PTH method is affected differently by preanalytical factors, conclusions should not be drawn about other PTH assays based on these results. Peripheral laboratories and clinicians conducting trials should be informed of the need to collect and store specimens according to protocols established at their laboratory lest results be affected and patient care be compromised.

We thank Amy Wong for assistance in the analysis of blood samples and organization of PTH data; we also thank the nurses in the hemodialysis units who facilitated the blood drawing.

References

1. Raisz LG, Kream BE, Lorenzo JA. Metabolic bone disease. In: Larsen PR, Kronenberg HM, Shlomo M, Polonsky KS, eds. Williams textbook of endocrinology, 10th ed. Philadelphia: WB Saunders, 2002:1373–410.
2. Polack MR, Yun ASL Clinical disturbances of calcium, magnesium, and phosphate metabolism. In: Brenner, BM, Rector FC, eds. The kidney, 7th ed. Philadelphia: WB Saunders, 2003:1041–51.
3. Martin KJ, Gonzáles EA, Slatopsky E. Renal osteodystrophy. In: Brenner BM, Rector FC, eds. The kidney, 7th ed. Philadelphia: WB Saunders, 2003: 2255–304.
4. Jane Ellis M, Livesey JH, Evans MJ. Hormone stability in human whole blood. Clin Biochem 2003;36:109–12.
5. Evans MJ, Livesey JH, Ellis MJ, Yandle TG. Effect of anticoagulants and storage temperatures on stability of plasma and serum hormones. Clin Biochem 2001;34:107–12.
6. Glendenning P, Laffer LL, Weber HK, Musk AA, Vasikaran SD. Parathyroid hormone is more stable in EDTA plasma than in serum. Clin Chem 2002;48:766–7.
7. Levin GE, Nisbet JA. Stability of parathyroid hormone-related protein and parathyroid hormone at room temperature. Ann Clin Biochem 1994;31:497–500.
8. Omar H, Chamberlin A, Walker V, Wood PJ. IMMULITE 2000 parathyroid hormone assay: stability of parathyroid hormone in EDTA blood kept at room temperature for 48 h. Ann Clin Biochem 2001;38:561–3.
9. Teal TK, Reed M, Stevens PE, Lamb EJ. Stability of parathyroid hormone ex vivo in haemodialysis patients. Ann Clin Biochem 2003;40:191–3.
10. Walker KS, Seth J. Stability of parathyroid hormone in blood from renal patients on haemodialysis. Ann Clin Biochem 2000;37:800–1.
11. Glendenning P, Musk AA, Taranto M, Vasikaran SD. Preanalytical factors in the measurement of intact parathyroid hormone with the DPC IMMULITE assay. Clin Chem 2002;48:566–7.
12. Scharnhorst V, Valkenburg J, Vosters C, Vader H. Influence of preanalytical factors on the Immulite intact parathyroid hormone assay. Clin Chem 2004;50:974–5.
13. Diagnostic Products Corporation. Reinstatement of EDTA as a sample type. Reference range and analytical sensitivity updates. Technical Bulletin #2068. Breda, The Netherlands: Diagnostic Products Corporation, 2004.
14. Blumsohn A, Al Hadari A. Parathyroid hormone: what are we measuring and does it matter? Ann Clin Biochem 2002;39:169–72.
15. Diagnostic Products Corporation Netherlands. IMMULITE 2000 intact PTH [Package Insert] Product No. PIL2KPP-12. Breda, The Netherlands: Diagnostic Products Corporation, 2004.
16. Bowen RA, Chan Y, Cohen J, Rehak NN, Hortin GL, Csako G, et al. Effect of blood collection tubes on total triiodothyronine and other laboratory assays. Clin Chem 2005;51:424–33.

DOI: 10.1373/clinchem.2004.046821

Anti-Actin Antibodies in Celiac Disease: Correlation with Intestinal Mucosa Damage and Comparison of ELISA with the Immunofluorescence Assay, *Antonio Carroccio,[1*] Ignazio Brusca,[2] Giuseppe Iacono,[3] Lidia Di Prima,[1] Saverio Teresi,[3] Giuseppe Pirrone,[1] Ada Maria Florena,[4] Stella Maria La Chiusa,[2] and Maurizio Rocco Averna[1]* ([1] Internal Medicine and [4] Pathology Department, University Hospital of Palermo, Palermo, Italy; [2] "Buccheri La Ferla" Hospital of Palermo, Palermo, Italy; [3] Pediatric Gastroenterology, "Di Cristina" Hospital of Palermo, Palermo, Italy; * address correspondence to this author at: Internal Medicine, University Hospital of Palermo, via del Vespro 141, 90127 Palermo, Italy; fax 39-091-6552936, e-mail acarroccio@hotmail.com)

The presence in the sera of celiac disease (CD) patients of anti-actin autoantibodies (AAAs) has been suggested as a

marker of severe intestinal villus atrophy *(1)*. AAAs have been detected with an immunofluorescence (IF) technique and seem to contribute to villus cytoskeleton damage and to the pathogenesis of intestinal damage in CD *(2)*.

The aims of the present study were to evaluate the relationship between the presence of serum IgA AAAs and severity of intestinal mucosa damage in CD patients and to compare the IF assay with a new ELISA for IgA AAA determination.

We enrolled 150 individuals in the study. IgA AAAs were assayed in 58 consecutive CD patients diagnosed between January and December 2003: 30 adults (10 male; median age, 32 years; range, 18–56 years) and 28 children (14 male; median age, 18 months; range, 1–12 years). The sera were collected at CD diagnosis, after overnight fasting, and were frozen at −80 °C for a mean of 9 months before AAA determination. In 20 patients, AAAs were reassayed after 6–12 months of gluten-free diet. CD diagnosis was based on the revised criteria of the European Society of Pediatric Gastroenterology and Nutrition *(3)*. We enrolled 64 patients as "healthy" controls [34 adults evaluated for suspected hypercholesterolemia (15 male; median age, 35 years; range, 18–56) and 30 children with recurrent pharyngotonsilitis (14 male; median age, 3 years; range, 1–12 years)]. None of these controls had symptoms or laboratory signs suggesting CD, and all were negative for anti-endomysium (EmA) and anti-transglutaminase (anti-tTG). We enrolled an additional 28 adults with autoimmune or gastrointestinal diseases other than CD as "disease" controls: type 1 autoimmune hepatitis (AH; 6 cases), type 2 AH (4 cases), systemic lupus erythematosus (4 cases), Sjögren disease (3), primary biliary cirrhosis (2), Crohn disease (4), multiple food intolerance (3), autoimmune enteropathy (1), and refractory sprue (1).

Finally, to test whether the anti-actin ELISA used was specific for F-actin, we assayed for IgG anti-F-actin in 18 sera from patients with type 1 AH, positive by IF for IgG anti-F-actin on HEP-2 cells (titer range, 1:160–1:1280), and 30 negative sera from healthy controls.

EmAs were assayed on human umbilical cord by IF *(4)*. The anti-tTG ELISA was performed with commercial reagents with recombinant human tTG as the antigen *(5)*.

Modified Hep-2 cells (HEP II actin; INOVA; ref. 508090) were incubated at room temperature for 30 min with serum serially diluted (1:10 to 1:1280) with phosphate-buffered saline (pH 7.2) in a covered moist chamber, washed twice in phosphate-buffered saline for 5 min, incubated at room temperature for 30 min, with fluorescein isothiocyanate-conjugated goat anti-human serum [anti-IgA; $F(ab')_2$ Anti-Human IgA; ref. 30240; Bio-Rad], washed twice as before, and read with a fluorescence microscope. The affinity-isolated rabbit AAAs were used as positive controls in all experiments (Sigma Chemical); rabbit monoclonal AAAs were used at a 1:50 dilution during the first incubation followed by a second incubation with the 1:100-diluted fluorescein isothiocyanate-conjugated anti-rabbit immunoglobulins (Sigma).

The IgA AAA enzyme immunoassay (ELISA) was performed with a commercially available method for anti-actin IgG determination (F-Actin Smooth Muscle; INOVA; ref. 708785) and an anti-serum anti-human IgA conjugate (INOVA; ref. 508549). We added 100 μL of diluted serum (1:101) to the wells and incubated for 30 min at room temperature. After the wells were washed three times with buffer, 100 μL of the IgA conjugate was added to each well, and the plates were incubated for 30 min and washed again. We added 100 μL of 3,3′,5,5′-tetramethylbenzidine (TMB) chromogen to each well and incubated the wells in the dark for 30 min at room temperature. Stopped reactions were read at 450 and 620 nm. The typical threshold value for AAA absorbance was arbitrarily fixed as equal to the mean value + 2 SD displayed by control sera (0.270). The control wells (no serum) introduced in each plate had an absorbance of <0.025. Intra- and interassay CV were calculated on 20 samples.

Three biopsy specimens of the second part of the duodenum were obtained *(5, 6)*; slides were stained with hematoxylin and eosin and graded by conventional histology according to Oberhuber et al. *(7)*.

Serum IgA AAAs evaluated by IF were positive in 54 of the 58 (93%; 95% confidence interval, 88–98%) untreated CD patients; samples from 3 CD children and 1 adult were negative. The titer of IgA AAA-positive samples ranged between 1:20 and 1:640 (median, 1:80). Serum IgA AAAs evaluated by ELISA were positive in samples from 51 of the 58 CD patients (86%; 79–93%); the samples from the same 4 patients negative by IF were also negative by ELISA, and samples from 3 additional CD children were negative. The correlation between AAA results by IF and ELISA was high: $r = 0.819$ (Spearman correlation coefficient; see Fig. 1 in the Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol51/issue5/). The within-assay imprecision (CV) of the ELISA method for IgA AAAs was 4.3%, and the between-assay CV was 9.2%.

None of the healthy controls had AAA values, as assayed by ELISA, higher than the cutoff, and values were significantly higher in CD patients [mean (SD), 0.974 (0.924)] than in controls [0.157 (0.057); Mann–Whitney test, $z = 6.4$; $P < 0.0001$]. Among the patients with autoimmune diseases or with various intestinal diseases, 10 had values above the cutoff: 6 patients with type 1 AH [all positive for anti-smooth muscle antibodies (ASMAs)]; 2 patients with primary biliary cirrhosis (both positive for anti-mitochondrial antibodies and 1 positive for ASMAs), and 2 patients with multiple food intolerance. The AAA values of CD patients were significantly higher than those of the disease controls (Mann–Whitney test, $P < 0.001$). Regarding the IF AAA assay results for the 2 control groups, there were 8 positives in the healthy controls (all with a titer of 1:20) and 9 positives in the disease controls (titers, 1:40–1:160), 7 of whom also were positive by ELISA.

Intestinal histology of the CD patients revealed that 8 had mild villus atrophy, 23 had marked villus atrophy, and 27 had total villus atrophy. All patients with marked

or total villus atrophy were positive for AAAs in the ELISA, whereas all 7 patients negative for AAA had mild villus atrophy. There was a significant inverse correlation between AAA values by ELISA and IF and the villus-height/crypt-depth ratio ($r = -0.447$ by ELISA, $r = -0.404$ by IF; $P < 0.0001$; Fig. 1). Furthermore, the CD patients negative for AAAs by ELISA had significantly higher villus/crypt ratios than the patients positive for AAAs [mean (SD), 1.41 (0.32) vs 0.52 (0.15); $P < 0.01$].

Anti-tTG values and EmA titer showed a significant, but lower, inverse correlation with severity of intestinal mucosa damage [Spearman correlation, $r = -0.324$ for anti-tTGs, $r = 0.315$ for EmAs). We found no correlation between anti-tTG and AAA values.

In 19 of the 20 CD patients, AAA assay results were negative, by both ELISA and IF, after 6–12 months on gluten-free diet.

IgG AAA results were positive by ELISA (absorbance range, 0.300–0.935) in 17 of the 18 patients with type 1 AH showing anti-F-actin antibodies with IF, whereas none of samples from healthy controls gave positive results.

Gluten ingestion in CD is associated with alterations of the intestinal mucosa and interactions between gliadin and several extracellular matrix components, leading to the formation of neo-epitopes, which act as autoantigens *(8, 9)*. AAAs, described in CD very recently *(1)*, are among the autoantibodies consequent to the formation of these autoantigens in CD patients.

Our results show that AAAs correlate with severity of intestinal villus damage in CD patients: The AAA ELISA was positive in 51 of the 58 CD patients studied, and all patients with severe intestinal mucosa damage were positive for AAAs. The 7 patients negative for AAAs had mild villus atrophy. Furthermore, there was a highly significant inverse correlation between AAA values (assayed both by ELISA and IF) and the villus/crypt ratios in CD patients. A similar correlation coefficient (0.56) between AAA titers and severity of intestinal mucosa lesions has been reported in a multicenter study on the usefulness of AAA assays in CD *(10)*.

Both AAA assays (IF and ELISA) were also positive in several disease controls, mainly in patients with type 1 AH and primary biliary cirrhosis. In general, the ELISA for IgA AAAs that we used in the present study showed good reproducibility and a higher sensitivity than the previously described ELISAs *(1, 11, 12)*, and its use can be recommended. This probably depends on the use of F-actin and not G-actin as antigen in the ELISA: In vitro incubation of intestinal epithelial cells with gliadin caused intracellular actin polymerization with an increase in F-actin *(2)*, which is the real neo-epitope recognized by AAAs. The major problem in using an F-actin ELISA is the difficulty of getting, in vitro, 100% of actin in its polymerized form (F-actin) and avoiding the unpolymerized form (G-actin), which could interfere with the quality of the test. However, the materials we used seemed to be specific for F-actin antibodies as 17 of the 18 patients with type 1 AH and none of the controls were IgG AAA-positive with the ELISA. Additional studies involving a greater number of anti-F-actin-positive patients are needed to confirm this result.

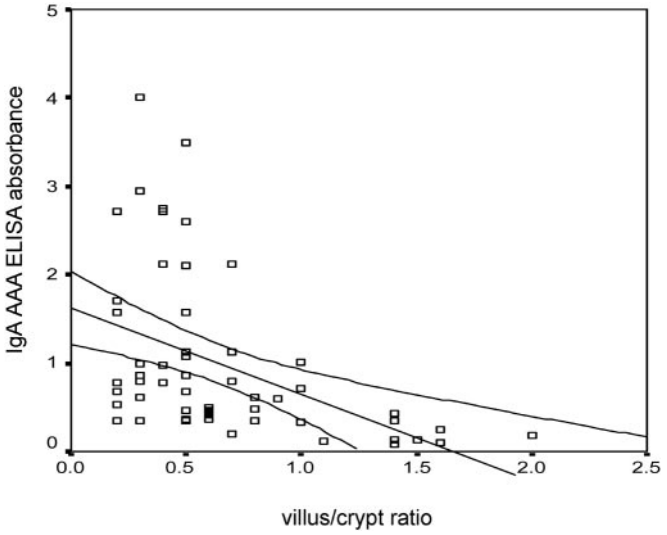

Fig. 1. Correlation between AAA values by ELISA, expressed as absorbance, and the villus/crypt ratio in 58 untreated CD patients.

In conclusion, AAAs are a reliable marker of severe intestinal mucosa damage in CD patients, and a simple ELISA technique offers an accurate method for their determination. We propose that, after exclusion of coexistent CD and type 1 AH, intestinal biopsy is not necessary for villus/crypt evaluation in EmA/AAA-positive patients.

We are indebted to Professor A. Craxì and his research group (Gastroenterology Division of Palermo University, Palermo, Italy), who provided the stored sera from patients suffering from type 1 AH. We thank Carole Greenall for excellent revision of the English. We are particularly grateful to M.G. Clemente (Pediatric Department, Cagliari University, Italy) for technical advice on the AAA IF assay. We also thank C. Scalici and G. Di Fede for assistance in revising the manuscript. This work was supported by a grant from the Ministry of Universities and Scientific Research (decr 86 Ric, dated January 30, 2002) and of the Ministry of Agriculture of Italy (DM 224/7303/02, dated June 10, 2002; project "ALICE").

References

1. Clemente MG, Musu MP, Frau F, Brusco G, Sole G, Corazza GR, et al. Immune reaction against the cytoskeleton in celiac disease. Gut 2000;47:520–6.
2. Clemente MG, De Virgilis S, Kang JS, Macatagney R, Musu MP, Di Pierro MR, et al. Early effects of gliadin on enterocyte intracellular signalling involved in intestinal barrier function. Gut 2003;52:218–23.
3. Walker-Smith JA, Guandalini S, Schmitz J, Shmerling DH, Visakorpi JK. Revised criteria for diagnosis of coeliac disease. Arch Dis Child 1990;65:909–11.
4. Carroccio A, Cavataio F, Iacono G, Agate V, Ippolito S, Kazmierska I, et al. IgA antiendomysial antibodies on the umbilical cord in diagnosing celiac disease. Sensitivity, specificity and comparative evaluation with the traditional kit. Scand J Gastroenterol 1996;31:759–63.
5. Carroccio A, Vitale G, Di Prima L, Chifari N, Napoli S, La Russa C, et al. Comparison of anti-transglutaminase ELISAs and anti-endomysial antibody

assay in the diagnosis of celiac disease: a prospective study. Clin Chem 2002;48:1546–50.
6. Carroccio A, Giannitrapani L, Soresi M, Not T, Iacono G, Di Rosa C, et al. Guinea pig transglutaminase immuno-linked assay does not predict celiac disease in patients with chronic liver disease. Gut 2001;49:506–11.
7. Oberhuber G, Granditsch G, Vogelsang H. The histopathology of coeliac disease: time for a standardized report scheme for pathologists. Eur J Gastroenterol Hepatol 1999;11:1185–94.
8. Verbeke S, Gotteland M, Fernandez M, Bremer J, Rios G, Brunser O. Basement membrane and connective tissue proteins in intestinal mucosa of patients with coeliac disease. J Clin Pathol 2002;55:440–5.
9. Stulik J, Hernychova L, Porkertova S, Pozler O, Tuckova L, Sanchez D, et al. Identification of new celiac disease autoantigens using proteomic analysis. Proteomics 2003;3:951–6.
10. Clemente MG, Musu MP, Troncone R, Volta U, Congia M, Ciacci C, et al. Enterocyte actin autoantibody detection: a new diagnostic tool in celiac disease diagnosis: results of a multicenter study. Am J Gastroenterol 2004;99:1551–6.
11. Zamanou A, Tsirogianni A, Terzoglu C, Balafas A, Economidou I, Lymberi P. Anti-smooth muscle antibodies (ASMAs) and anti-cytoskeleton antibodies (ACTAs) in liver diseases: a comparison of classical indirect immunofluorescence with ELISA. J Clin Lab Anal 2002;16:194–201.
12. Granito A, Muratori F, Cassani F, Pappas L, Muratori D, Agostinelli L, et al. Anti-actin IgA antibodies in severe coeliac disease. Clin Exp Immunol 2004;137:386–92.

DOI: 10.1373/clinchem.2004.041665

Handling of and Direct Sampling from Primary Barcode-Labeled Pediatric Tubes on Vitros Clinical Chemistry Analyzers Integrated into an enGen Work Cell, *Ayşe Y. Demir, Wouter W. van Solinge, and Hans Kemperman*[*] (Department of Laboratory Medicine, University Medical Center Utrecht, Utrecht, The Netherlands; * address to correspondence to this author at: Department of Laboratory Medicine, University Medical Center Utrecht, HP G.03.550, PO 85500, 3508 GA Utrecht, The Netherlands; fax 31-30-2505418, e-mail h.kemperman@azu.nl)

Handling and directly sampling from primary barcode-labeled pediatric tubes is a challenge for laboratories receiving substantial numbers of samples from pediatric patients *(1)*. To prevent iatrogenic anemia from frequent blood draws, preanalytical as well as analytical systems should be capable of handling small sample volumes collected in pediatric tubes *(2)*. However, most analyzers and robotic systems have been developed for standard 5- to 7-mL test tubes and cannot handle small pediatric tubes. This incompatibility leads to manual processing of pediatric samples and transfer of samples to micro cups even in large automated laboratories. Manual handling of specimens and transfer of samples are laborious and may lead to errors in both the preanalytical and analytical phases.

In most hospitals, blood of pediatric patients is collected in small pediatric tubes by heel puncture, in contrast to adult patients from whom blood is collected by venipuncture in 5- to 7-mL evacuated tubes. Although most instruments require that the barcode label be applied vertically for reading, regular barcode labels do not conveniently fit on small pediatric tubes *(2)*. Because of the reduced size of the tubes, separate buckets are needed for centrifugation, and often the barcode labels, which are too large for the small tubes, interfere with proper fitting in the buckets; they can also get damaged and no longer be readable *(1)*. Furthermore, standard analyzer sample racks and trays do not support the dimensions of pediatric tubes. The same holds for tube carriers used by various work cells and track systems.

In our clinical chemistry department, we receive ~2500 test tubes daily from various departments of the University Medical Center in Utrecht, The Netherlands. Among these tubes, ~500 pediatric tubes are derived from the pediatric hospital that is part of our organization. Until recently, these tubes were analyzed in a separate satellite laboratory in the pediatric hospital. For efficiency reasons, chemistry tests previously performed in the pediatric hospital, ~250 tubes per day, were moved to our central laboratory, in which two Vitros 950 and one Vitros 250 dry chemistry analyzers (Ortho Clinical Diagnostics) are integrated into an enGen™ work cell (Thermo Electron Corporation). This work cell includes an entry/exit module where samples can be loaded and unloaded. A robotic arm picks up the tubes from sample blocks placed in 1 of the 3 entry drawers and puts each tube in an individual carrier. The barcode is read, and the information is written to a chip in the base of the carrier. The computer uses the requested tests and the status of the different analyzers to calculate an optimal route for each tube through the work cell to the analyzers. Tubes with a cap are first routed to the decapper. After completion of all requested tests, the tubes are sent to the entry/exit module again, where the tubes are placed in barcode-labeled storage racks. These storage racks can be placed directly in the refrigerator. If a tube is needed, for example for additional tests, the data manager will identify at which position in which block the tube is located.

To be able to handle pediatric tubes through the whole preanalytical and analytical phase in a way similar to that for the standard 5- to 7-mL tubes, our laboratory has made the following adjustments. Before blood drawing at the pediatric hospital, 500-μL Capiject lithium-heparin pediatric gel tubes (Terumo Capiject System; Omnilabo) are prefixed in Microtainer tube extenders (Becton Dickinson; Fig. 1A), which make handling of the tubes during blood drawing easier. More importantly, the tube extenders make possible uniformity in size of both pediatric and adult specimens. As a result, the regular barcode labels can be placed in the usual vertical position on the primary pediatric tubes and be transported in standard racks (Fig. 1B). The pediatric tubes are centrifuged together with the standard tubes in the same buckets and centrifuge. Because the sample probe senses the liquid height and starts sampling just below the surface, the Vitros analyzers can deal with variable liquid heights in different sample tubes. However, to enable pipetting directly from these primary barcode-labeled pediatric tubes, the speed at which the sample probe is lowered had to be increased because the fluid height drops more rapidly in these tubes during aspiration as a result of the smaller inner diameter.