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Comparison of 7 Methods for Extracting Cell-Free DNA from Serum Samples of Colorectal Cancer Patients

To the Editor:

The presence of cell-free DNA of tumor origin in serum or plasma of cancer patients *(1)* has triggered numerous studies to explore the diagnostic and prognostic potential of circulating DNA. This DNA, present only in minute concentrations in plasma or serum, is highly fragmented *(2)*, a condition that often leads to substantial loss of DNA of small sizes in the course of DNA isolation. The lack of consensus regarding which extraction method is better for the efficient capture of such DNA might be partially responsible for the large disparities in the literature, which are reflected in reports of total concentrations of plasma or serum DNA alone *(3)* or DNA integrity measurement as a diagnostic or prognostic tool. The ability to detect mutated v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (*KRAS*) DNA in serum has been reported to vary with the chosen DNA isolation methods *(4)*.

We evaluated in parallel 7 isolation approaches (Table 1) by extracting cell-free DNA from 12 pooled sera obtained from 67 colorectal cancer patients and grouped on the basis of TNM tumor staging [tumor extent (T), spread to lymph nodes (N), and metastasis (M)].[1] The approaches we evaluated involved diverse strategies for DNA isolation. The experimental set-up involved DNA isolation from a 2-mL aliquot of serum in duplicate followed by DNA quantification by the fluorescent Quant-iT dsDNA HS assay (Invitrogen) and a Taqman real-time PCR (rPCR) technique on the cadherin 1, type 1, E-cadherin (epithelial) (*CDH1*) gene. To exclude false results in the *CDH1* amplification due to various PCR inhibitors present in DNA extracts, the isolated DNA was further quantified by Taqman rPCR on bisulfite-converted DNA because the procedure of bisulfite-conversion of DNA removes many PCR-inhibitory components such as proteins, EDTA, and ethanol (EpiTech bisulfite kit, Qiagen). A fragment devoid of the CpG site of the actin, beta (*ACTB*) gene was the target for this round of rPCR. All 3 assays for DNA quantification were carried out in duplicate, and the DNA/gene amount in samples was interpolated by reference to corresponding standard curves generated by using 5 to 6 serial dilutions of a DNA standard. Appropriate blanks were included in each run. For rPCR, a positive control sample was run in each plate to control the interplate variation.

The 7 extraction methods showed remarkable differences in the recovery of DNA from serum (Table 1). The phenol-chloroform procedure (PCI-glycogen), sodium iodide method (NaI method), and QIAamp DNA blood kit generated significantly higher yields of DNA, assessed by fluorescent measurement, than the other 4 methods (all $P < 0.05$). Assessed by rPCR targeting on *CDH1* (amplicon size: 68 bp), the NaI method was ranked top in the list, and statistical significance ($P < 0.05$) was achieved in all pairwise comparisons except with the PCI-glycogen approach. The latter, however, outperformed only the guanidine-resin procedure as determined by application of the Dunnett T_3 test for pairwise multiple comparisons (Table 1). Because of the insufficient balance of DNA in the other 2 samples, we carried out the second round of rPCR on bisulfite-converted DNA in 10 pooled samples. Among 3 methods evaluated, the NaI method exhibited the most abundant gene copy numbers of *ACTB* (amplicon size: 115 bp), with the median of *ACTB* being 7 times higher than that generated by the QIAamp DNA blood kit, although the difference did not reach the statistical significance (Table 1).

The higher recovery of DNA obtained with the NaI and PCI glycogen procedures was also revealed on the agarose gel, which showed much stronger DNA signals along each of 2 lanes (electrophoretic image available on request). Interestingly, substantial amounts of small DNA fragments were recovered with these 2 methods, a result that was not achievable with the other 5 protocols (Table 1). Furthermore, the size of these small fragments appeared to correspond to that of nucleosomal DNA, i.e., approximately 180–220 bp or its multiples. Because circulating DNA is highly fragmented, any isolation method that favors capture of fragmented DNA from serum or plasma will be useful for a variety of downstream applications in modern clinical and translational research laboratories. These include efficient detection of mutations and capture of DNA methylation markers from serum or plasma.

In comparing the PCI-glycogen approach and the NaI method, we found that the latter was not only superior to the former in terms of DNA quantity, as assessed by 2 rounds of rPCR, but also was simpler, more rapid, and less costly (data available on request).

To summarize, the results of the present study, which involved 7 isolation methods performed via

[1] A total of 67 Chinese patients with sporadic colorectal cancers who underwent potentially curative surgical resection at the Department of Colorectal Surgery, Singapore General Hospital, between February 2004 and November 2005, participated in the present study. None of these patients had a known history of familial adenomatous polyposis, hereditary nonpolyposis colorectal cancer, or other types of cancers. None of them received preoperative chemotherapy or radiotherapy. The institutional review board of Singapore General Hospital approved this study, and all study participants provided written informed consent.

Table 1. Yield and fragment size of serum DNA isolated by 7 methods.

	DNA yield from 2 mL of serum						Visible bands on electrophoresis from 1 mL of serum		
	By fluorescent assay, ng		By rPCR on *CDH1* (relative amount to a positive control)		By rPCR targeting *ACTB* on BS[a] templates (relative amount to a positive control)				
Extraction method	n	Median (SE)	n	Median (SE)	N	Median (SE)	≅200 bp	≅400 bp	≅500 bp
PCI-glycogen[b]	12	367.958 (94.645)[c]	12	332.220 (119.031)[d]	10	15.507 (8.127)	Yes	Yes	Yes
NaI method[e]	12	306.040 (61.228)[c]	12	391.735 (89.558)[f]	10	17.476 (9.915)	Yes	Yes	Yes
Guanidine-resin method[g]	6	8.928 (0.364)	12	1.145 (0.352)		ND	No	No	Yes
QIAamp DNA Blood Midi kit with carrier RNA[h]	12	228.915 (38.162)[c]	12	69.934 (14.869	10	2.247 (2.601	No	No	Yes
ChargeSwitch 1-mL serum kit[i]	12	83.165 (13.370)	12	74.978 (16.612)		ND	No	No	Yes
ZR serum DNA kit[j]	6	15.363 (6.580)	12	6.577 (5.749)		ND	No	No	Yes
Puregene DNA purification System Cell and Tissue Kit[k]	12	59.200 (11.652)	12	42.094 (19.447)			No	No	Yes

[a] BS, bisulfite-converted; ND, not detected.
[b] Phenol-chloroform method with addition of glycogen.
[c] DNA yield was significantly higher than that isolated by the methods without any mark in the column ($P < 0.05$, by Friedman analysis followed by Dunnett T_3 test for pairwise multiple comparisons), but there was no difference among marked methods.
[d] *CDH1* quantity was second highest among all 7 methods but statistical significance was reached only in pairwise comparison with the guanidine-resin method ($P < 0.05$).
[e] Sodium iodide method (protocol available on request).
[f] *CDH1* quantity was highest among all 7 methods and of statistical significance in all pairwise comparisons except with the PCI-glycogen procedure ($P < 0.05$).
[g] Adapted from the method described by Wang et al. *(4)*.
[h] Performed according to manufacturer's instructions except with addition of 3 μg of carrier RNA into each sample; catalog no. 51183, lot no. 127136152.
[i] Performed according to manufacturer's instructions, catalog no. CS 11040, lot no. 1409017.
[j] Performed according to manufacturer's instructions, catalog no. D3013, lot no. 32-200607019.
[k] Performed according to manufacturer's instructions, part no. D-5500A, lot no. GS18781.

quantification of serum DNA by 3 assays and examination of fragment sizes of DNA isolated by electrophoresis, indicated that the NaI procedure consistently revealed better performance. Therefore, this procedure appears to be a suitable method for cell-free DNA extraction for many downstream applications in cancer research.

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Cross-Reactivity of Phentermine with an Immunoassay Designed to Detect Amphetamine in a Meconium Specimen

To the Editor:

Neonates exposed to drugs of abuse in utero can experience prenatal drug dependence leading to withdrawal symptoms and a number of other health problems *(1)*. Early detection of exposure is critical to guide necessary treatment and improve outcomes for these children. Meconium begins to form in the digestive tract at 12–16 weeks gestation. Drugs and metabolites collect in meconium beginning at about 5 months gestation. Thus, meconium testing can identify exposure to drugs during the last 4 months of a full-term pregnancy *(2)*.

Our laboratory uses ELISA reagents (Immunalysis) to detect drugs of abuse in meconium. Poor specificity of immunoassay reagents for amphetamines is well characterized and as a result, specimens that test positive for amphetamines by immunoassay are routinely tested by a second analytical method to prevent false-positive results. Our ELISA screen for meconium has separate detection antibodies for amphetamine and methamphetamine. The ELISA cutoff for these drugs is 20 ng/g. All positive screen results are confirmed by GC-MS.

We report the investigation of an unconfirmed positive amphetamine result. ELISA assay of the meconium specimen in question was positive for amphetamine but negative for methamphetamine. The confirmation assay failed to detect amphetamine, methamphetamine, 3,4-methylenedioxyamphetamine, or 3,4-methylenedioxy-*N*-methylamphetamine. The specimen was then analyzed with a more comprehensive liquid-chromatography–tandem mass-spectrometry method to scan for possible candidates that could elicit a positive screen result.

Briefly, 0.25 g of meconium was extracted with 1 mL of 25 mmol/L phosphate buffer, pH 7.3, and centrifuged. Solid-phase extraction on a 250-μL aliquot was perfomed with a mixed-mode column. Deuterated analogs were added as internal standards. The drugs were eluted with 2 mL of methanol: ammonium hydroxide (98:2 vol:vol), evaporated to dryness under nitrogen, and reconstituted in 50 μL methanol. Analyses were performed with an Agilent Technologies 1200 series liquid chromatograph coupled to a 6410 triple quadrupole mass spectrometer operated in the positive electrospray mode and equipped with a Zorbax Eclipse XDB C18 column (4.6×50 mm, 1.8 μm) at 40 °C and an ammonium-acetate:methanol mobile phase. The specimen was found to contain 191 ng/g phentermine, an anorexiant stimulant recommended for short-term use in the treatment of obesity.

We quantified phentermine by using d5-methamphetamine as the internal standard. Because the molecular weight of methamphetamine and phentermine are the same, the transitions for liquid chromatography–tandem mass spectrometry were also the same, so the compounds were separated on the basis of retention time (methamphetamine 5.9 min; phentermine 6.6 min). Two transitions were selected: The quantifying transition was *m/z* 150.2–91.1 and the qualifying transition was *m/z* 150.2–65.1. Ion ratios were within ±20% to meet the criterion for a positive result. The monitored transition for the d5-methamphetamine was *m/z* 155.2–92.1.

The ELISA kit insert indicates that phentermine has 89% cross-reactivity at 25 ng/g with the amphetamine antibody, but does not cross-react with the methamphetamine antibody. Phentermine was fortified at 3 concentrations (100, 200, and 400 ng/g) and run as unknowns in the amphetamine and methamphetamine ELISA. The phentermine-fortified samples showed changes in binding (B/B0) of 44%, 29%, and 16% in the amphetamine assay, whereas the methamphetamine assay showed negligible change in binding (B/B0) at 88%, 86%, and 85%. B/B0 is defined as the absorbance reading of the sample (B) divided by the absorbance reading of the zero-dose standard (B0). The change in B/B0 for phentermine obtained by using both kits is shown in Fig. 1. Although phentermine and methamphetamine have the same molecular weight of 149 Da, they differ in the position of the additional methyl group. Therefore, coupling of the antibody through the aromatic ring of amphetamine, rather than the nitrogen, minimizes cross-reactivity with methamphetamine but still allows cross-reactivity with the amphetamine antibody.

To the best of our knowledge, this is the only reported incidence of the confirmation and quantification of phentermine in a meconium specimen. Phentermine is a pregnancy category C drug, indicating it should not be administered during pregnancy unless clearly needed because no data are available to determine if it adversely affects the fetus. Although no evidence of toxicity or

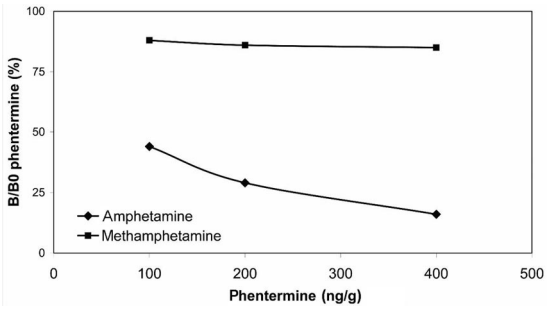

Fig. 1. Change in binding (B/B0) of phentermine to amphetamine and methamphetamine ELISA antibody.

lethality was observed in rat pups from administering of phentermine to dams *(3)*, 5 adverse outcomes (stillbirths) occurred in a previous study of 118 pregnant women taking phentermine *(4)*. This case demonstrates the importance of performing confirmatory testing for drugs of abuse and supports a need to include phentermine in confirmatory testing to distinguish its use or abuse from that of amphetamine or other related drugs.

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New Highly Sensitivity Assay Used to Measure Cardiac Troponin T Concentration Changes During a Continuous 216-km Marathon

To the Editor:

We recently measured cardiac troponin T (cTnT)[1] concentrations serially in blood from 10 participants of the Badwater ultramarathon, a continuous 216-km race that takes place under extreme environmental conditions. Details on the Badwater ultramarathon itself, and on the training experience and health condition of the athletes have been reported previously *(1)*. Briefly, the athletes had completed a mean of 43.4 (median 20, range 5–130) marathons and 20.4 (median 15, range 6–80) ultramarathons, and the mean finishing times for the Badwater race were 51.24 h (median 45.2 h, range 43.5–61.4 h). cTnT was measured with a new highly sensitive assay for cardiac troponin T (Hs-TnT) (Roche Diagnostics) on an ELECSYS 2010 automated analyzer that uses chemiluminescence technology. As described previously *(2)*, the interassay CV of this assay is 8% at 10 ng/L and 2.5% at 100 ng/L, and the intraassay CV is 5% at 10 ng/L and 1% at 100 ng/L. The diagnostic range of this assay is 2 to 10000 ng/L.

Blood samples (EDTA plasma) were drawn at baseline, after the first half marathon, after each full marathon distance, and at the finish. In a previous study we tested the third-generation cTnT assay in the same study population and were unable to detect cTnT concentrations above the lower limit of detection in any blood sample before or during the

[1] Nonstandard abbreviations: cTnT, cardiac troponin T; Hs-TnT, highly sensitive assay for cardiac troponin T; BNP, brain natriuretic peptide.

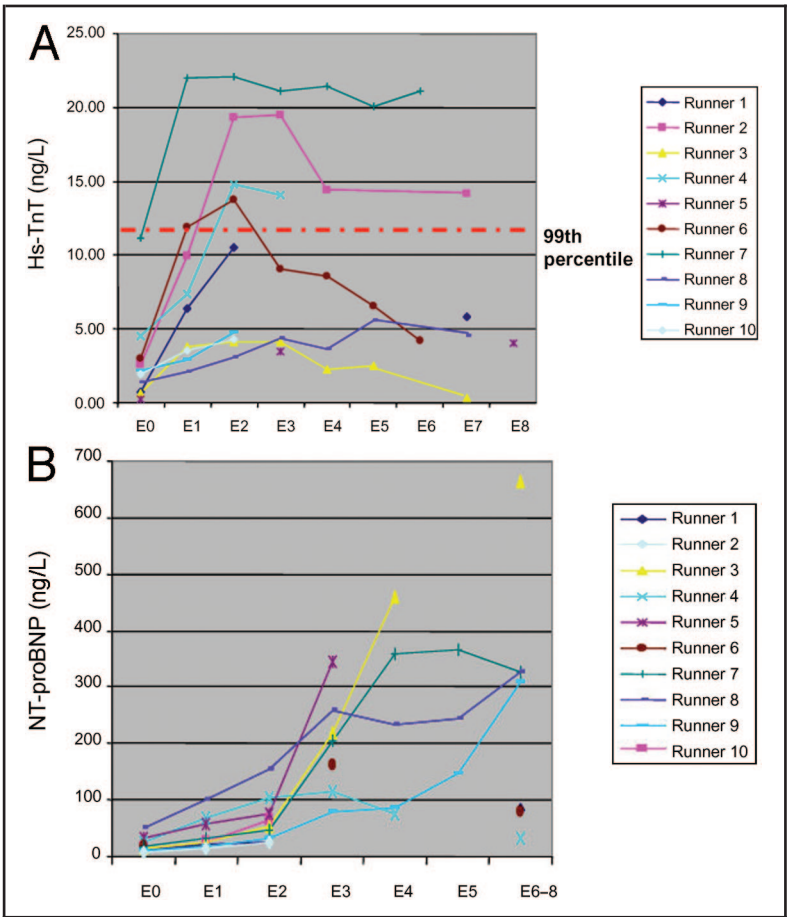

Fig. 1. Concentrations of Hs-TnT (A) and N-terminal pro-BNP (NT-proBNP) (B) in individual runners during a continuous 216-km ultramarathon.

E0 to E8 denote the time points of blood sampling: E0, half-marathon distance; E1, full-marathon distance; E3, double-marathon distance; E4, quadruple-marathon distance; E7, finish; E8, shortly after E7.

ultramarathon *(1)*. We now report that with the new Hs-TnT assay, cTnT was measurable in all samples including the baseline samples. The time course of individual Hs-TnT values is displayed in Fig. 1A. We observed several patterns of cTnT concentration changes detected with over time with the Hs-TnT assay, including cases with constantly low concentrations without changes as well as cases with an rise of Hs-TnT early after the start of strenuous exercise. In one case, Hs-TnT decreased to a low preexercise concentration while the athlete was still running. The latter case is extremely interesting because this observation suggests a physiological counterregulatory process rather than a simple increase of myocardial damage related to the intensity of exercise. It is tempting to speculate that such an adaptation mechanism could be meaningful in limiting the magnitude of myonecrosis. Previous studies on endurance athletes suggested that training intensity and the type of exercise were major determinants of the rate and magnitude of subsequent troponin release *(3, 4)*. A mechanism that in our opinion may contribute to this adaptation mechanism is release of brain natriuretic peptide (BNP). In animal models, cytoprotective and growth-regulating effects of BNP have been reported *(5)*. BNP has been shown to open adenosine-triphosphate–sensitive potassium channels of myocardial mitochondria via the natriuretic peptide receptor A signaling pathway. Furthermore, BNP exerts counterregulatory and sympathoinhibitory effects inhibiting hypertrophy in cultured cardiac myocytes and angiotensin-II–stimulated collagen synthesis by cardiac fibroblasts. Supporting this hypothesis, in our study population N-terminal pro-BNP, the biologically inactive cleavage product of pro-BNP, increased in 9 of 10 runners (inadequate sampling in 1 case), and peak values of N-terminal pro-BNP were significantly higher (424 vs 126 ng/L, $P = 0.0063$) in those ultraendurance runners who demonstrated an increase of cardiac troponin above the 99th percentile value as measured with the new more sensitive and precise troponin T assay (Fig. 1B). Although a potential limitation of this study is that blood sampling was not complete in all cases, a consistent change supported by 2 sequential samples was present for the most important observations. Assessment of right and left ventricular performance and hemodynamics would have been informative but was not available in this cohort. Owing to the small number of individuals studied and the heterogeneity of the release patterns, confirmatory follow-up studies are needed. Nevertheless, the observation is interesting and has allowed generation of a hypothesis that should be validated in future studies.

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AQP4-IgG Immunoprecipitation Assay Optimization

To the Editor:

The sensitivity and specificity of tissue-based immunofluorescence assays (IFA)[1] is established for detecting neuromyelitis optica (NMO) IgG as an aid to classifying inflammatory demyelinating central nervous system disorders belonging to the NMO spectrum *(1)*: NMO, partial and inaugural forms of NMO [longitudinally extensive transverse myelitis (≥3 vertebral segments radiologically) and recurrent optic neuritis] and pediatric inflammatory autoimmune encephalopathies. Immunoprecipitation assays (IPA) employing recombinant human aquaporin-4 (AQP4), the antigen of NMO, have been described *(2, 3)*. In optimizing the green fluorescent protein (GFP)-tagged AQP4 IPA *(2)* for high-throughput testing, we encountered cases in which patient IgG bound to GFP and not to AQP4. Here we report our efforts, in a clinical setting, to determine the rate of false positivity and take steps to eliminate it.

From October 1, 2007 to March 31, 2008, the Mayo Clinic Neuroimmunology Laboratory evaluated, by use of GFP-AQP4 IPA, sera from 117 healthy adult control individuals (group 1) and from 5500 patients for whom service NMO-IgG IFA testing was requested. Of these, 557 were IFA-positive (group 2) and 4943 were IFA negative (group 3). We also tested neurologically asymptomatic patients, 58 with hypergammaglobulinemia (group 4) and 27 with, systemic lupus erythematosus or Sjögren syndrome (group 5).

In the course of clinical serological evaluation for NMO-IgG (indirect immunofluorescence assay performed on a service basis in the Mayo Clinic Department of Laboratory Medicine and Pathology), patients were identified. Medical records were reviewed retrospectively. The Institutional Review Board (IRB) of the Mayo Clinic College of Medicine, Rochester, MN, approved the study (IRB 07-007453).

We added 30 μL of serum to duplicate 85-μL aliquots of recombinant human GFP-AQP4 [150 000 GFP counts, solubilized in lysis buffer (700 mmol/L NaCl, 10 mmol/L Tris-HCl pH 8.0, 1 mmol/L EDTA, 0.5% Triton X-100)] from human embryonic kidney–293 cells transfected with pEGFP-AQP4 (plasmid-DNA–encoding enhanced GFP-AQP4) *(2)* and held 16 h at 4 °C. Control specimens contained 4 different pools of normal human serum samples or 1 serum sample

[1] Nonstandard abbreviations: IFA, immunofluorescence assay; NMO, neuromyelitis optica; IPA, immunoprecipitation assays; AQP4, human aquaporin-4; GFP, green fluorescent protein; MuSK, muscle-specific kinase; IRB, Institutional Review Board.

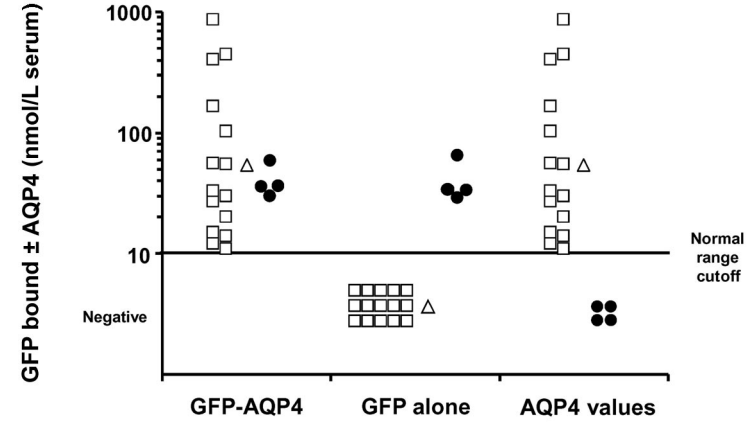

Fig. 1. Frequency of false-positive AQP4 immunoprecipitation in 20 patients (group 3) who were negative for NMO-IgG by IFA but positive by GFP-AQP4 immunoprecipitation assay, and for whom clinical information was available.

Subtraction of values for GFP alone eliminated 4 patients (false positives, 5%) with diagnoses: □, NMO-spectrum disorder; △, single-episode optic neuritis; ●, (corrected values negative) neuroretinitis, primary progressive multiple sclerosis, neurosarcoidosis, or paraneoplastic optic neuropathy.

from an AQP4-IgG–positive patient. Two hours after adding protein-G sepharose 4B (30 μL; Zymed Laboratories), we washed the beads 6 times with lysis buffer and measured GFP spectrophotometrically (excitation, 485 nm; emission, 535 nm). After subtracting the maximum value yielded by normal control sera, we calculated GFP-AQP4 bound (nmol/L serum) by reference to a GFP standard (Clontech). Sera yielding values >10 nmol/L serum were clarified by centrifugation and reassayed with GFP-AQP4 and with GFP alone (150 000 counts). The final results represent each patient's mean GFP-AQP4 value after subtracting the individual's value for precipitation of GFP alone.

Analysis of serum from groups 1, 4 and 5 yielded a final GFP-AQP4 concentration ≤10 nmol/L serum. In group 2, of 557 sera positive by IFA, none precipitated GFP alone, and 331 yielded initial and finally corrected AQP4-IgG concentrations >10 nmol/L (59%; median 46.6, range 10.1–3661 nmol/L). Clinical information was available for 54 of these 331 patients (16%). All had an NMO-spectrum disorder: 28 patients had NMO, 24 had longitudinally extensive transverse myelitis (recurrent in 12 patients), and 2 had recurrent optic neuritis. In group 3, of 4943 sera that were NMO-IgG negative by IFA, 80 yielded initial AQP4-IgG concentrations >10 nmol/L (1.6%; Fig. 1); 76 remained positive after GFP values were subtracted (median 32.6, range 10.2–867 nmol/L). Diagnoses for the 4 patients who were finally seronegative after GFP values were subtracted (AQP4-IgG range 29–65 nmol/L) were neuroretinitis, multiple sclerosis, neurosarcoidosis, and paraneoplastic optic neuropathy. Clinical information was available for 16 of the 76 patients who remained seropositive. Fifteen (93.8%) had an NMO-spectrum disorder [NMO, 3; longitudinally extensive transverse myelitis, 10 (recurrent in 5 patients); recurrent optic neuritis, 2] and 1 had monophasic optic neuritis.

The possibility of false-positive results for AQP4-IgG due to immunoprecipitation of GFP must be considered when sera are evaluated by using recombinant autoantigens fused with this tag. This consideration is of particular importance in patients who are seronegative for NMO-IgG by standardized IFA (group 3 in our study). Among 80 such patients, we obtained a false-positive rate of 5%. After we eliminated those patients from consideration, all but 1 of the remaining 16 patients who had serum samples positive by IPA alone and for whom clinical information was available were confirmed to have a clinical disorder recognized to be in the NMO spectrum. The exceptional seropositive patient had a diagnosis of monophasic optic neuritis. One can reasonably speculate that seropositivity in this case predicts future relapse. False-positive results analogous to those we report for the GFP-AQP4 assay have been reported in immunoprecipitation assays for the muscle nicotinic acetylcholine receptor antibody (^{125}I-α-bungarotoxin tag) *(4)* and muscle-specific kinase (MuSK) antibody (placental alkaline phosphatase tag) *(5)*. The results of this large study suggest that our optimized high-throughput IPA using GFP-AQP4 is not as sensitive as the originally described tissue-based IFA for detecting NMO-IgG. However, as an adjunct to IFA, the IPA described here enhances the diagnostic yield of NMO-spectrum disorders. A more formal comparison of sensitivity and specificity of IFA, IPA, and combined assays in consecutively-acquired patients with clinical information available in each case is underway. Assurance of assay specificity requires elimination of false-positive results to preclude misdiagnosis and inappropriate long-term commitment of patients to immunotherapies with attendant expense and risks.

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Comparison of Bromcresol Purple and Capillary Protein Electrophoresis for Quantification of Serum Albumin in Multiple Myeloma

To the Editor:

According to the International Staging System *(1)*, serum albumin concentration is a key factor in determining patient prognosis in multiple myeloma. A recent study published in *Clinical Chemistry* described significant discordance between bromcresol green (BCG)[1] albumin dye and agarose gel serum protein electrophoresis (SPEP) for albumin concentration determination in patients with high concentrations of monoclonal (M)-protein *(2)*. Also available, however, are commonly used alternative methods for albumin determination, such as bromcresol purple (BCP) albumin dye and capillary zone SPEP *(3)*. To examine whether the previously observed comparative discordance of measured albumin concentration is limited only to the BCG dye and agarose gel SPEP methods of albumin determination *(2)*, we retrospectively compared measured albumin concentrations as determined by BCP albumin dye and capillary zone SPEP in a large cohort of multiple myeloma patients.

The ratio of measured BCP albumin to SPEP albumin concentrations in patients with a previously identified monoclonal protein was retrospectively calculated for 579 specimens submitted to the University of Arkansas Immunology laboratory for routine serum protein electrophoresis by capillary zone SPEP. All specimens had concurrent albumin determination by a BCP albumin dye method. These consisted of 355 IgG, 122 IgA, 29 IgM, 13 IgD, and 60 free–light-chain myeloma patient specimens. The monoclonal protein light chains in these specimens were approximately evenly divided between κ and λ. Serum albumin was measured by the Beckman-Coulter Synchron BCP assay, and total serum protein was determined by using the Beckman-Coulter Synchron biruet method on a Beckman-Coulter LX20 Promodular system. A Sebia Capillarys 2 was used to determine the relative serum sample albumin and M-protein fractions. The relative albumin and M-spike fractions were multiplied by total protein to obtain final SPEP albumin and M-protein concentrations.

Substantial discrepancies in albumin determination in the presence of M-protein between capillary zone SPEP and the BCP albumin determination methods were observed. Although albumin

[1] Nonstandard abbreviations: BCG, bromcresol green; SPEP, serum protein electrophoresis; BCP, bromcresol purple; M, monoclonal.

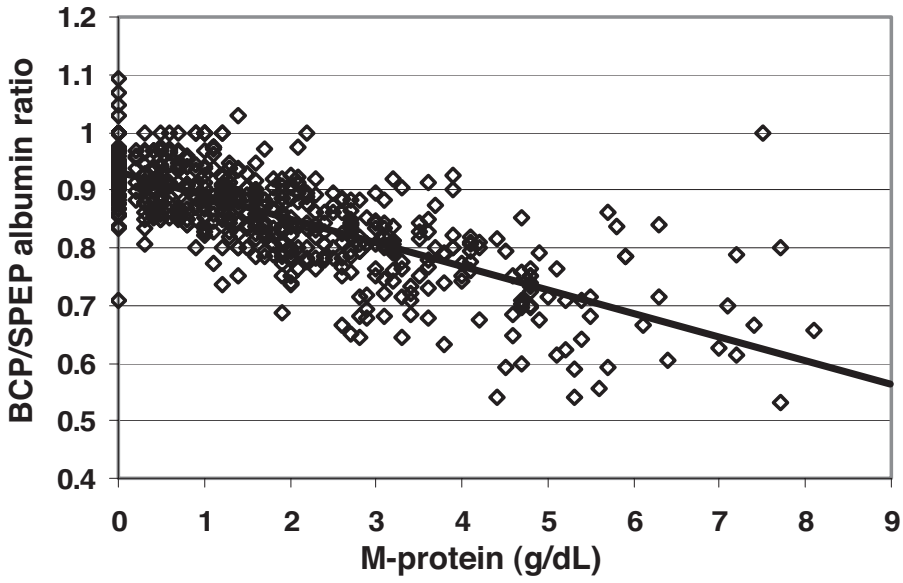

Fig. 1. Ratio of BCP albumin concentration measurement to capillary SPEP albumin concentration measurement vs M-protein concentration as determined by capillary SPEP analysis for all 579 specimens used in this study.
The best-fit Deming regression line is shown.

concentrations similar to those measured by BCP and capillary SPEP were observed in patients with undetectable concentrations of an M-protein, albumin concentrations reported by capillary SPEP were consistently higher than albumin concentrations determined by BCP in the presence of M-protein. When the ratio of BCP albumin/SPEP albumin vs M-protein was plotted (Fig. 1) the effect appeared to be linear. Deming regression fit of all sample measurements yielded the following equation: (ratio of BCP/SPEP albumin) = −0.041 (M-protein in g/dL) + 0.93, $R^2 = 0.55$. The 95% CI of the slope was −0.036 to −0.045 and was significantly different from a slope of 1 ($P < 0.001$, calculated with CBstat version 5.1). Negative slopes for BCP/SPEP albumin ratios were also observed for IgG (−0.045 g/dL), IgA (−0.032 g/dL), IgM (−0.040 g/dL), and IgD (−0.032 g/dL) specimens. Too few free light-chain samples with measurable SPEP M-protein concentrations >0.2 g/dL (2 g/L) were present to determine if albumin ratios were affected by the presence of free light chains alone.

The differences between capillary electrophoresis and BCP albumin dye measurements presented here are similar to those previously observed for BCG dye and agarose electrophoresis albumin determinations, demonstrating that this discrepancy is not limited to specific types of bromcresol albumin dyes or electrophoretic albumin measurements In both studies, increasing amounts of M-protein led to relatively higher albumin measurements by electrophoresis compared to albumin determined with serum dye. Although the mechanism for this effect merits further investigation, the observation of relatively higher albumin results in both capillary electrophoresis (which detects proteins by ultraviolet absorbance) and agarose gel electrophoresis disfavors models in which the observed discrepancy is caused by nonlinear staining of monoclonal protein *(2)*. This study, however, cannot rule out other potential models, including nonlinear detection of strong M-protein bands by ultraviolet absorbance, nonlinear and/or nonequivalent measurement of different protein species in the kinetic biruet total-protein determination assay used *(4)*, or interference by M-protein in the BCP albumin dye assay.

In the absence of M-protein, the BCP albumin measurement was approximately 93% of that obtained by capillary SPEP, whereas BCG yielded higher measurements than those obtained by agarose gel electrophoresis *(2)*. This result may be partially explained by a positive bias of BCG to BCP albu-

min measurements previously observed in our laboratory.

In summary, the presence of 5 g/dL (50 g/L) of M-protein results in BCP albumin determinations that are approximately 30% lower than those obtained using capillary SPEP. According to the International Myeloma Staging System, samples with albumin values of <3.5 g/dL (35 g/L) can potentially be classified as stage I in the absence of increased serum β_2-microglobulin. Here, 521 samples had albumin concentrations <3.5 g/dL (35 g/L) by BCP assay, whereas only 374 samples exhibited albumin concentrations <3.5 g/dL (35 g/L) by capillary electrophoresis. Because the Multiple Myeloma International Staging System does not specify a preferred method for albumin determination, clinicians should be aware of these differences in methods when interpreting albumin concentrations in myeloma patient samples *(1, 5)*.

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Changes in Solvent Composition in Tandem Mass Spectrometry Multiplex Assay for Lysosomal Storage Disorders Do Not Affect Assay Results

To the Editor:

Lysosomal storage disorders (LSDs) embody a collection of >40 unique genetic diseases that cause the accumulation of macromolecular substrates normally degraded by lysosomal (and in some cases, nonlysosomal) enzymes involved in lysosomal metabolism *(1)*. Although individual LSDs are rare, their combined incidence has been estimated at 1 per 7700 live births *(1)*. Recently, 2 reports that describe the use of a tandem mass spectrometry–based multiplex assay for newborn screening for LSDs have been published in *Clinical Chemistry (2, 3)*. A third report describes the availability of QC dried blood spot materials for monitoring the quality of the multiplex assay for Krabbe (galactocerebrosidase), Gaucher (acid β-glucocerebrosidase), Niemann-Pick types A and B (acid sphingomyelinase), Pompe (acid α-glucosidase), and Fabry (acid α-galactosidase) disorders *(4)*. The multiplex assay has been shown to be robust and is currently in use or under evaluation in many screening and diagnostic laboratories for newborns around the world.

Current protocols call for the use of acetonitrile as a major component of the mobile phase. Acetonitrile is a widely used solvent in newborn-screening laboratories for LSD and other mass spectrometry–based assays (e.g., for amino acids and acylcarnitines). Several chemical suppliers have informed their customers of a worldwide acetonitrile shortage, which may severely impact all tandem mass spectrometry–based assays conducted in newborn-screening laboratories around the world. To

maintain the continuity of current LSD newborn-screening efforts, we evaluated the use of methanol as an alternative solvent to acetonitrile in the multiplex assay described by Zhang et al. *(2)*.

HPLC-grade methanol was purchased from Fisher Scientific and used as received. The mobile phase and reconstitution solvent consisted of 0.2% formic acid in methanol/water (volume ratio, 80:20). The last step of the assay entails reconstituting samples in 200 μL of the mobile phase and introducing the samples into the mass spectrometer via flow injection. Analysis at the CDC was conducted on an API 3200 instrument (Applied Biosystems) as previously described *(4)*. We prepared 16 dried blood spots of LSD QC material exhibiting typical-to-low activities of lysosomal enzymes. Samples were assayed in duplicate according to our protocols, divided into 2 aliquots, and then evaporated in separate 96-well plates before their introduction into the mass spectrometer. One plate was reconstituted with the previously described mobile phase, and the second plate was reconstituted with the methanol-substituted solution. The samples for all 5 disorders were analyzed according to our standard operating procedure, as previously reported *(4)*. The mean (SD) enzyme activities for the acetonitrile-based assay were 2.31 (0.19) μmol · L^{-1} · h^{-1} for galactocerebrosidase, 6.81 (0.58) μmol · L^{-1} · h^{-1} for acid β-glucocerebrosidase, 3.06 (0.36) μmol · L^{-1} · h^{-1} for acid sphingomyelinase, 16.5 (1.25) μmol · L^{-1} · h^{-1} for acid α-glucosidase, and 7.64 (0.63) μmol · L^{-1} · h^{-1} for acid α-galactosidase. The mean enzyme activities for the methanol-based assay were 2.32 (0.17) μmol · L^{-1} · h^{-1} for galactocerebrosidase, 6.77 (0.76) μmol · L^{-1} · h^{-1} for acid β-glucocerebrosidase, 3.03 (0.41) μmol · L^{-1} · h^{-1} for acid sphingomyelinase, 16.7 (1.14) μmol · L^{-1} · h^{-1} for acid α-glucosidase, and 7.41 (0.44) μmol · L^{-1} · h^{-1} for acid α-galactosidase (Fig. 1). No statistically significant differences were observed among the samples analyzed for this study, and the overall variation between sample set means was <5%, well within the assay's imprecision as described by Zhang et al. *(2)*.

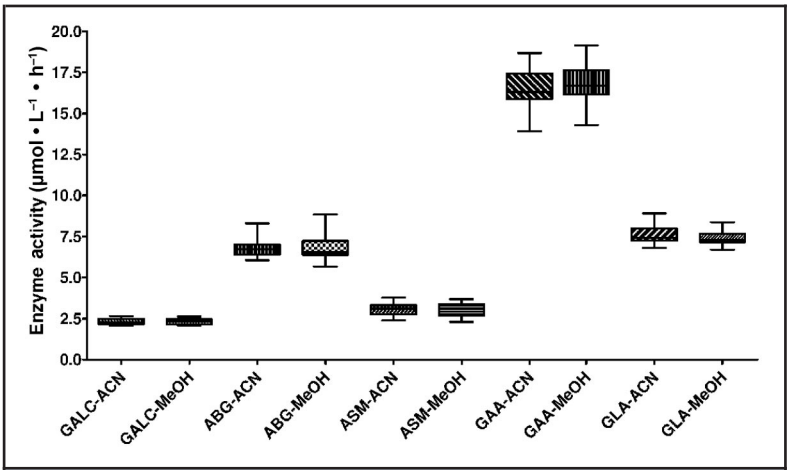

Fig. 1. Effects of substituting methanol (MeOH) for acetonitrile (ACN) in the mobile phase on the multiplex tandem mass spectrometry assay for 5 LSD diseases.

GALC, galactocerebrosidase (Krabbe disease); ABG, acid β-glucocerebrosidase (Gaucher disease); ASM, acid sphingomyelinase (Niemann-Pick types A and B); GAA, acid α-glucosidase (Pompe disease); GLA, acid α-galactosidase (Fabry disease).

These results strongly suggest that HPLC-grade methanol may be substituted for acetonitrile in the LSD multiplex assay without deleterious effects on instrument performance or sample results. Although we found different mobile phases to have very similar ion intensities (data not shown), we did not evaluate different methanol grades and suppliers. We recommend that users refer to Annesley's report on matrix effects associated with electrospray ionization *(5)* to understand the potential differences between methanol products from different manufacturers before effecting a change in solvent for the LSD assay. Laboratories interested in changing the assay protocol should conduct a thorough validation study to recognize the effects of their choice of solvent grade and supplier, as well as their specific equipment's analytical performance. The QC materials developed and distributed by the CDC may be used for this purpose. Any efforts to evaluate the multiplex LSD assay by newborn-screening laboratories worldwide should not be hindered by the unavailability of acetonitrile, which may become a large issue in the near future.

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