HDL particle aggregates after precipitation of apo B-containing lipoproteins.

The data on HDL particle size obtained in this study (mean diameter, 8.8 nm) are in agreement with the values described in the literature for other techniques. In healthy normolipidemic individuals, HDL sizing by nuclear magnetic resonance spectroscopy yielded diameters of ~9.2 nm *(10)*, whereas the values obtained from gradient gel electrophoresis ranged from 8.4 to 9.6 nm *(9, 13)*. In this study, similar to other published results obtained by gradient gel electrophoresis *(13)*, the HDL particle diameter (nm) was greater in premenopausal women than in men (9.1 vs 8.4 nm; Mann–Whitney test, $P = 0.013$). Negative correlations between HDL particle size and total cholesterol and LDL-cholesterol were also reported by Pascot et al. *(13)*, thus strengthening the link between HDL particle size and coronary artery disease. In the present study, the correlations between HDL particle size and HDL-cholesterol and triglyceride concentrations were not statistically significant, probably because of the small number of samples. Pascot et al. *(13)* may have found significant correlations for these relationships because they studied >400 individuals. Therefore, LLS analysis after chemical precipitation of apo B-containing lipoproteins gave results for HDL diameter in the same range as those obtained by established techniques for lipoprotein sizing *(9, 10, 13)*. Furthermore, the differences between genders and the correlations reported here are in agreement with those described in the literature *(13)*.

Because LLS analysis after chemical precipitation is a practical and less time-consuming approach for HDL sizing, it could be used in large trials and in routine clinical laboratory analysis.

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### References

1. Barter P, Kastelein J, Nunn A, Hobbs R. High density lipoproteins (HDLs) and atherosclerosis; the unanswered questions. Atherosclerosis 2003;168: 195–211.
2. Skinner ER. High-density lipoprotein subclasses. Curr Opin Lipidol 1994;5: 241–7.
3. Lamarche B, Moorjani S, Cantin B, Dagenais GR, Lupien PJ, Després JP. Associations of $HDL_2$ and $HDL_3$ subfractions with ischemic heart disease in men. Prospective results from the Quebec Cardiovascular Study. Arterioscler Thromb Vasc Biol 1997;17:1098–105.
4. Johansson J, Carlson LA, Landou C, Hamsten A. High density lipoproteins and coronary atherosclerosis. A strong inverse relation with the largest particles in confined to normotriglyceridemic patients. Arterioscler Thromb 1991;11:174–82.
5. Tangney CC, Mosca LJ, Otvos JD, Rosenson RS. Oral 17b-estradiol and medroxyprogesterone acetate therapy in postmenopausal women increases HDL particle size. Atherosclerosis 2001;155:425–30.
6. Syvanne M, Ahola M, Lahdenperii S, Kahri J, Kuusi T, Virtanen KS, Taskinen MR. High density lipoprotein subfractions in non-insulin-dependent diabetes mellitus and coronary artery disease. J Lipid Res 1995;36:573–82.
7. Moriguchi EH, Tamachi H, Goto Y. Hepatic lipase activity and high-density lipoprotein in hypercholesterolemia familial: adaptational mechanisms for LDL-receptor deficient state. Tokai Exp Clin Med 1990;15:401–6.
8. Warnick RG, Nauck M, Rifai N. Evolution of methods for measurement of HDL-cholesterol: from ultracentrifugation to homogeneous assays. Clin Chem 2001;47:1579–96.
9. Pérusse M, Pascot A, Deprés JP, Couillard C, Lamarche B. A new method for HDL particle sizing by polyacrylamide gradient gel electrophoresis using whole plasma. J Lipid Res 2001;42:1331–4.
10. Soedamah-Muthu SS, Chang YF, Otvos J, Evans RW, Orchard TJ. Lipoprotein subclass measurements by nuclear magnetic resonance spectroscopy improve the prediction of coronary artery disease in type 1 diabetes. A prospective report from the Pittsburgh Epidemiology of Diabetes Complications Study. Diabetologia 2003;46:674–82.
11. O'Neal D, Harrip P. Dragicevic G, Rae D, Best JD. A comparison of LDL size determination using gradient gel electrophoresis and light-scattering methods. J Lipid Res 1998;39:2086–90.
12. Dias VC, Parsons HG, Boyd NG, Keane P. Dual-precipitation method evaluated for determination of high-density lipoprotein (HDL), HDL2 and HDL3 cholesterol concentrations. Clin Chem 1988;34:2322–7.
13. Pascot A, Lemieux I, Bergeron J, Tremblay A, Nadeau A, Prud'homme D, et al. HDL particle size: a marker of the gender difference in the metabolic risk profile. Atherosclerosis 2002;160:399–406.

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**Quantitative Assessment of *PML-RARa* and *BCR-ABL* by Two Real-Time PCR Instruments: Multiinstitutional Laboratory Trial,** *Pascual Bolufer,[1*] Dolors Colomer,[2] Maria T. Gomez,[3] Joaquín Martínez,[4] Silvia M. Gonzalez,[3] Marcos Gonzalez,[5] Josep Nomdedeu,[6] Beatriz Bellosillo,[7] Eva Barragán,[1] Francesco Lo-Coco,[8] Daniela Diverio,[9] Lourdes Hermosin,[10] José García-Marco,[11] Maria D. de Juan,[12] Francisco Barros,[13] Rafael Romero,[14] and Miguel A. Sanz,[15] for the Group of Molecular Biology in Hematology* ([1] Molecular Biology, Department of Medical Biopathology, Hospital Universitario La Fe, Avda Campanar 21, 46009 Valencia, Spain; [2] Hematopathology Unit, Hospital Clínic, Barcelona, Spain; [3] Molecular Biology, Hematology, Hospital Gran Canaria Dr. Negrin, Las Paslmas de GC, Spain; [4] Molecular Biology, Hematology, Hospital 12 de Octubre, Madrid, Spain; [5] Immunopathology and Molecular Biology, Hematology, Hospital Clínico Universitario, Salamanca, Spain; [6] Laboratory of Hematology, Hospital de la Santa Creu i Sant Pau, Barcelona, Spain; [7] Laboratory of Cytogenetics and Molecular Biology, Service of Pathology, Hospital del Mar, Barcelona, Spain; [8] Laboratory of Integrated Diagnosis of Oncohematologic Diseases, University Tor-Vergata, Rome, Italy; [9] Laboratorio di Diagnostica Molecolare Oncoematologica, Dipartimento di Biotecnologie Cellulari ed Ematologia, Universitá degli Studi "La Sapienza", Rome, Italy; [10] Biology, Hematology, Hospital de Jerez, Jerez de la Frontera, Cádiz, Spain; [11] Molecular Cytogenetic Unit, Servicio de Hematologia, Hospital Universitario Puerta de Hierro, Madrid, Spain; [12] Unified Laboratory, Immunology, Hospital Donostia, San Sebastian, Gupuzcoa, Spain; [13] Molecular Medicine Unit-INGO (Sergas), University of Santiago de Compostela, Hospital Clínico Universitario de Santiago, Santiago de Compostela, Spain; [14] Departamento de Estadistica e Investigación Operativa, Universidad Politécnica de Valencia, Valencia, Spain; [15] Clinical Hematology, Service of Hematology, Hospital Universitario La Fe, Hospital Universitario La Fe, Valencia, Spain; * author for correspondence: fax 34961973030, e-mail bolufer_pas@gva.es)

The recent introduction on the market of instruments for real-time PCR has prompted the development of quanti-

**Table 1. Results of the *PML-RARa* and *BCR-ABL* assays (copies/μL) for the participating laboratories grouped according to the instrument.**

| | | Laboratories | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | LC | | | | | | | ABI | | | |
| Assay | Samples | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
| *BCR-ABL* | Blank | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 1 | 5 | 1 | 2 | 1 | 1 | 1 | 0 | 1 | 1 | 3 | 1 |
| | 2 | 22 299 | 13 120 | 16 370 | 6204 | 26 430 | 19 503 | 14 300 | 10 667 | 12 455 | 23 353 | 12 691 |
| | 3 | 10 | 15 | 12 | 3 | 29 | 16 | 47 | 8 | 13 | 27 | 9 |
| | 4 | 3 | 2 | 5 | 1 | 4 | 2 | 3 | 1 | 3 | 5 | 2 |
| | 5 | 6 | 1 | 2 | 1 | 2 | 4 | 18 | 1 | 1 | 4 | 1 |
| | 6 | 12 | 4 | 5 | 3 | 6 | 7 | 13 | 4 | 4 | 15 | 5 |
| | 7 | 178 | 60 | 86 | 41 | 122 | 131 | 282 | 11 | 13 | 249 | 110 |
| | 8 | 31 | 11 | 18 | 8 | 27 | 27 | 106 | 65 | 121 | 43 | 13 |
| | 9 | 2 | 2 | 3 | 2 | 2 | 3 | 22 | 1 | 1 | 2 | 1 |

| | | Laboratories | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Replicates | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| *PML-RARa* | | | | | | | | | | | |
| BCR1 | Blank | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | Blank | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 1 | 858 | 2825 | 1255 | 1965 | 1536 | 1758 | 1329 | 919 | 1291 | 2193 |
| | 1 | 675 | 3165 | 1195 | 1892 | 1776 | 2071 | 1342 | 752 | 1023 | 2400 |
| | 2 | 66 | 241 | 116 | 125 | 150 | 138 | 128 | 76 | 121 | 218 |
| | 2 | 90 | 284 | 142 | 231 | 145 | 132 | 136 | 97 | 133 | 194 |
| | 3 | 13 | 22 | 20 | 6 | 13 | 23 | 11 | 15 | 16 | 23 |
| | 3 | 13 | 40 | 13 | 2 | 12 | 18 | 13 | 6 | 13 | 22 |
| | 4 | 2 | 4 | 1 | 2 | 2 | 5 | 1 | 1 | 2 | 1 |
| | 4 | 2 | 1 | 1 | 2 | 1 | 3 | 2 | 2 | 1 | 1 |
| BCR3 | Blank | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | Blank | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 5 | 302 | 349 | 747 | 378 | 583 | 427 | 531 | 444 | 875 | 493 |
| | 5 | 329 | 323 | 609 | 611 | 706 | 585 | 509 | 399 | 842 | 507 |
| | 6 | 31 | 31 | 57 | 74 | 42 | 36 | 44 | 46 | 100 | 48 |
| | 6 | 34 | 31 | 61 | 56 | 47 | 34 | 43 | 48 | 89 | 45 |
| | 7 | 5 | 3 | 8 | 4 | 4 | 5 | 5 | 4 | 16 | 5 |
| | 7 | 7 | 3 | 7 | 6 | 7 | 5 | 5 | 7 | 13 | 7 |
| | 8 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 2 | 0 |
| | 8 | 1 | 0 | 5 | 0 | 0 | 1 | 1 | 1 | 1 | 0 |

tative assays for the most common fusion transcripts detectable in hematologic malignancies. However, because the ABI PRISM apparatus (ABI; Applied Biosystems) was the first available instrument for real-time PCR, most of the methods developed for the ABI PRISM use TaqMan probe chemistry *(1–3)*. With the introduction of other real-time PCR instruments, such as the LightCycler (LC; Roche), other methods have been described *(4–7)*. The instruments differ in several respects, including the light sources and the approach to acquisition of fluorescence data. Few reports have compared the results obtained with different types of real-time PCR instruments *(8)*. To the best of our knowledge, no such multicenter studies with common calibrators and common methods have been reported.

In the present study we analyzed the results obtained with two of the more widely used instruments for real time PCR, i.e., the ABI and LC, for amplifying two rearrangements frequently detectable in human leukemia, the *BCR-ABL* and *PML-RARa* fusion genes. For *BCR-ABL* several quantitative methods have been established for both instruments *(3–7)*, whereas for *PML-RARa* most of the quantitative methods have been developed for the ABI PRISM *(1)*.

The quantification of *BCR-ABL* transcripts is clinically relevant for monitoring patients with chronic myeloid leukemia undergoing allogeneic hematopoietic stem cell transplantation *(4, 9)* or treatment with interferon-a or imatinib mesylate *(9–11)*. For example, low numbers of *BCR-ABL* transcripts after 2 weeks of imatinib treatment predict a good response to imatinib after 4 weeks *(9)*. With respect to *PML-RARa*, recent reports have shown that quantitative assessment of *PML-RARa* transcripts allowed efficient monitoring of minimal residual disease

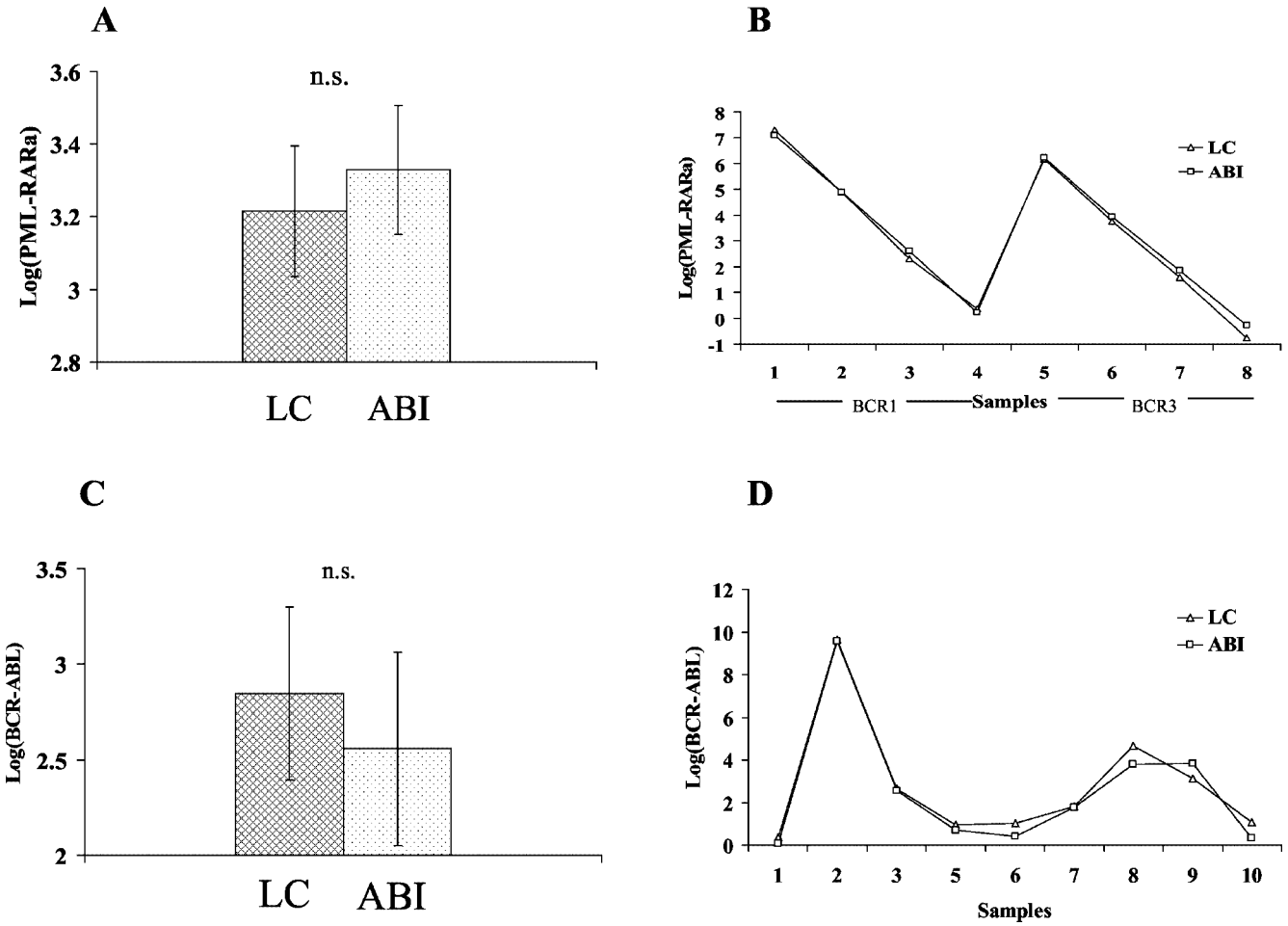


Fig. 1. Pooled means and profiles of means for the samples.

(*A*), pooled means of log(*PML-RARa*) for the LC and ABI. The *error bars* indicate 2 SD. (*B*), profiles of the means of the *PML-RARa* samples analyzed by each type of real-time PCR instrument. (*C*), pooled means of log(*BCR-ABL*) for the LC and ABI. The *error bars* indicate 2 SD. (*D*), profiles of the means of the *BCR-ABL* samples processed by each type of real-time PCR instrument. *n.s.*, not significant.

*(1)* and assessment of the effects of the treatment given *(12)*. Furthermore, patients who had transcription values above an empirical checkpoint after consolidation therapy had an increased risk of relapse *(13)*.

Ten laboratories participated in the trial for *PML-RARa* analysis (6 using LC and 4 ABI), and 11 laboratories were involved in the *BCR-ABL* assay (7 using LC and 4 ABI; see the list of participating laboratories in the Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol50/issue6/). In the trial for *PML-RARa*, each laboratory received 20 samples: 2 reagent blanks, 4 BCR1-positive, and 4 BCR3-positive cDNA samples in two replicates. In the trial for *BCR-ABL*, the laboratories received 10 samples: 1 blank and 9 patient cDNA samples (5 B3A2-positive and 4 B2A2-positive).

The laboratories also received calibrators prepared by cloning PCR products of samples from positive patients (B3A2 isoform for *BCR-ABL*, and BCR1 or BCR3 isoforms for *PML-RARa*) into CR® II-TOPO® vector (TOPO™ Cloning® Kit). For *PML-RARa*, two sets of calibrators were prepared: one for BCR1 and the other for the BCR3 isoform. Calibrators were provided at the following concentrations: $2 \times 10^5$, $2 \times 10^4$, $2 \times 10^2$, $2 \times 10^1$, and 2 copies/$\mu$L. Samples and calibrators were shipped on dry ice by overnight courier and stored refrigerated until used. The calibrators were analyzed in duplicate and the samples in triplicate.

For *PML-RARa* quantification, the laboratories equipped with ABI used the reagents and protocol established by Gabert et al. *(14, 15)* for this instrument in the Europe Against Cancer Program (EAC Protocol April 2002). The method designed for the ABI PRISM was optimized in the LC by use of a final volume of 10 $\mu$L, including 2 $\mu$L of cDNA samples (unknowns) or 2 $\mu$L of calibrators (for the calibration curve; see the Methods file in the online Data Supplement).

For the *BCR-ABL* rearrangement the laboratories equipped with ABI used the reagents and protocol designed by Gabert et al. *(14, 15)*. The ABI method was also optimized for the LC as described (see the Methods file in the online Data Supplement).

The quantification of transcripts was carried out automatically with the software provided in each type of equipment (see the section on quantification in the Methods file in the online Data Supplement).

To compare the results among the laboratories or samples for each real-time instrument, we performed a multivariate ANOVA for laboratories and samples as described in the Statistics file in the online Data Supplement.

The efficiencies estimated from the slopes of the calibration curves for the *PML-RARa* BCR1 and BCR3 isoforms for the ABI and LC were very similar (Table 1 in the online Data Supplement). One false-positive result was detected in a BCR1 blank sample (Table 1).

All laboratories detected transcripts in the replicate of the BCR1-positive sample with the lowest number of copies, and three of the four ABI and two of the seven LC laboratories detected the replicate sample BCR3 with the lowest number of copies (Table 1). These results suggest that both instruments are capable of detecting 1–5 copies/$\mu$L of the *PML-RARa* isoforms.

We observed significant differences among global means of the *PML-RARa* results of the laboratories with LC or ABI ($P = 0.000$). However, the global means of all samples assessed with LC or ABI showed no statistical difference (Fig. 1A), as reflected in the profiles of the means of the samples processed by each real-time instrument (Fig. 1B).

We found a difference in the interaction reproducibility between the two instruments at the limits of statistical significance ($P \approx 0.05$; Table 2 in the online Data Supplement), which was attributable to the larger variance of the LC for this component.

The efficiencies estimated from the slope of the calibration curves for *BCR-ABL* were similar for the two instruments [mean (SD) 1.79 (0.04) for the LC and 1.84 (0.02) for the ABI; Table 1 in the online Data Supplement]. No false-positive results were reported for the blank controls (Table 1).

All participating laboratories except one using the LC were able to detect transcripts in the sample with the lowest amount of *BCR-ABL* transcripts (1–5 copies/$\mu$L; Table 1).

We observed significant differences among the global means of the laboratories using LC or ABI ($P < 0.001$). Conversely, we found no statistically significant differences between the ABI and LC instruments for the global means of pooled samples of the laboratories (Fig. 1C). In addition, the profiles of the means of log(*BCR-ABL*) obtained for each sample within the laboratories using an ABI or LC instrument were nearly identical (Fig. 1D).

None of the precision components differed between the ABI and LC (Table 2 in the online Data Supplement).

In summary, this multicenter study showed that the ABI and LC instruments performed similarly. As a multilaboratory trial, the results obtained can be expected to better transfer to the data reported in clinical trials than if the comparison were performed by a single laboratory. The study confirms the ability of the LC to use the TaqMan technology *(6, 16)* as an alternative to the HybProbes technology originally developed for this system *(5, 17)*.

In this study, the main statistical differences were among the pooled means of the data. These individual differences among the laboratories with the same instrument could be attributable to variability in the stability of control samples or reagents, methodologic proficiency, or instrument maintenance.

The small difference in the interaction reproducibility ($P \approx 0.05$) for *PML-RARa* could reflect a difference in sensitivity of LC to the influence of noncontrolled effects.

In conclusion, despite differences in reagents in our study, the results for the LC and ABI instruments were equivalent with respect to the means and precision, suggesting that the choice of instrument has little to do with results when laboratories use the same methods and calibrators. Standardization of quantitative real-time PCR studies in a multiinstitutional context will require adoption of common methods and calibrators. The harmonization of the results should in turn allow better comparison of data obtained in different therapeutic trials.

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## References


1. Cassinat B, Zassadowski F, Balitrand N, Barbey C, Rain JD, Fenaux P, et al. Quantitation of minimal residual disease in acute promyelocytic leukemia patients with t(15;17) translocation using real-time RT-PCR. Leukemia 2000;14:324–8.
2. van der Velden VHJ, Hochhaus A, Cazzaniga G, Szczepanski T, Gabert J, Van Dongen JJM. Detection of minimal residual disease in hematologic malignancies by real-time quantitative PCR: principles, approaches, and laboratory aspects. Leukemia 2003;17:1013–34.
3. Mensik E, Van De Loch A, Schattenberg A, Linders E, Schaap N, Van Kesssel G, et al. Quantitation of minimal residual disease in Philadelphia chromosome positive chronic myeloid leukaemia patients using real-time quantitative RT-PCR. Br J Haematol 1998;102:768–74.
4. Elmaagacli AH, Freist A, Hahn M, Opalka B, Seeber S, Schaefer UW, et al. Estimating the relapse stage in chronic myeloid leukaemia patients after allogeneic stem cell transplantation by the amount of BCR-ABL fusion transcripts detected using a new real-time polymerase chain reaction method. Br J Haematol 2001;113:1072–5.
5. Bolufer P, Sanz GF, Barragán E, Sanz MA, Cervera J, Lerma E, et al. Rapid quantitative detection of BCR-ABL transcripts in chronic myeloid leukemia patients by real-time reverse transcriptase polymerase-chain reaction using fluorescently labeled probes. Haematologica 2000;85:1248–54.
6. Eckert C, Landt O, Taube T, Seeger K, Beyermann B, Proba J, et al. Potential of LightCycler for quantification of minimal residual disease in childhood acute lymphoblastic leukemia. Leukemia 2000;14:316–23.
7. Emig M, Saussele S, Wittor H, Weisser A, Reiter A, Willer A, et al. Accurate and rapid analysis of residual disease in patients with CML using specific fluorescent hybridization probes for real time quantitative RT-PCR. Leukemia 1999;13:1825–32.
8. Exner MM, Lewinski MA. Sensitivity of multiplex real-time PCR reactions, using the LightCycler and the ABI PRISM 7700 Sequence Detection System, is dependent on the concentration of the DNA polymerase. Mol Cell Probes 2002;16:351–7.
9. Scheuring UJ, Pfeifer H, Wassmann B, Bruck P, Atta J, Petershofen EK, et al. Early minimal residual disease (MRD) analysis during treatment of Philadelphia chromosome/BCR-ABL-positive acute lymphoblastic leukemia with the Abl-tyrosine kinase inhibitor imatinib (STI571). Blood 2003;101:85–90.
10. Wang L, Pearson K, Pillitteri L, Ferguson JE, Clark RE. Serial monitoring of BCR-ABL by peripheral blood real-time polymerase chain reaction predicts the marrow cytogenetic response to imatinib mesylate in chronic myeloid leukaemia. Br J Haematol 2002;118:771–7.
11. Stentoft J, Pallisgaard N, Kjeldsen E, Holm MS, Nielsen JL, Hokland P. Kinetics of BCR-ABL fusion transcript levels in chronic myeloid leukaemia

patients treated with STI571 measured by quantitative real-time polymerase chain reaction. Eur J Haematol 2001;67:302–8.
12. Visani G, Buonamici S, Malagola M, Isidori A, Piccaluga PP, Martinelli G, et al. Pulsed ATRA as single therapy restores long-term remission in PML-RARα-positive acute promyelocytic leukemia patients: real time quantification of minimal residual disease. A pilot study. Leukemia 2001;15:1696–700.
13. Gallagher RE, Yeap BY, Bi W, Livak KJ, Beaubier N, Rao S, et al. Quantitative real-time RT-PCR analysis of PML-RARa mRNA in acute promyelocytic leukemia: assessment of prognostic significance in adult patients from intergroup protocol 0129. Blood 2003;101:2521–8.
14. Gabert J, Beillard E, Van Der Velden VHJ, Bi W, Grimwade D, Pallisgaard N, et al. Standardization and quality control studies of "real-time" quantitative reverse transcriptase polymerase chain reaction of fusion gene transcripts for residual disease detection in leukemia—A Europe Against Cancer Program. Leukemia 2003;17:2318–57.
15. Gabert J. RQ-PCR for early diagnosis of relapse risk in leukemia (Europe Against Cancer Program). http://ifrjr.nord.univ-mrs.fr/mrd_leukemia (accessed March 2002).
16. Kreuzer KA, Lass U, Bohn A, Landt O, Schmidt CHA. LightCycler technology for the quantitation of bcr/abl fusion transcripts. Cancer Res 1999;59:3171–4.
17. Barragan E, Bolufer P, Moreno I, Martin G, Nomdedeu J, Brunet S, et al. Quantitative detection of AML1-ETO rearrangement by real-time RT-PCR using fluorescently labeled probes. Leuk Lymphoma 2001;42:747–56.

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**Size-Selective Extraction of Peptides from Urine for Mass Spectrometric Analysis,** *Glen L. Hortin,[1*] Bonnie Meilinger,[1] and Steven K. Drake[2]* (Departments of [1] Laboratory Medicine and [2] Critical Care Medicine, Warren Magnuson Clinical Center, NIH, Bethesda, MD; * address correspondence to this author at: Department of Laboratory Medicine, NIH, Bldg 10, Room 2C-407, Bethesda, MD 20892-1508; fax 301-402-1885, e-mail ghortin@mail.cc.nih.gov)

Protein excretion in urine has been suggested as an indicator of kidney disease since the time of Hippocrates. In the early 1800s, Bright further established approaches for studying proteinuria as a marker for kidney disease *(1)*. As methods for quantitative and qualitative analysis have become more sophisticated, it has become possible to detect earlier stages of kidney disease and to differentiate different patterns of protein excretion *(1–3)*. Quantitative immunoassays of selected urinary components such as $\alpha_1$-microglobulin, albumin, IgG, and $\alpha_2$-macroglobulin have been shown to be useful in characterizing the nature of proteinuria *(4)*. Two-dimensional electrophoresis has provided a method for simultaneous analysis of numerous proteins in urine *(5, 6)*. Recently, a new dimension has been added to analysis of urinary components by mass spectrometric techniques, which detect many small peptide components below the size resolution of electrophoresis *(7, 8)*. The highly complex mixtures of small peptides in urine offer the potential for information-rich patterns for clinical diagnosis. Concentrations of urinary peptides serve not only as markers for kidney function but also as markers of other systemic physiologic processes. As examples, immunoassays for specific peptides provide measures of thrombosis and fibrinolysis *(9, 10)* and endocrine function *(11)*.

In the present study, we sought to identify a simple method to prepare urine specimens for the analysis of small peptide components. Sample preparation represents one of the major challenges for analysis of peptide components in urine specimens by mass spectrometry. Ideally, sample preparation needs to accomplish three tasks: (*a*) concentration of relatively dilute peptide components; (*b*) removal of salts that suppress peptide ionization in mass spectrometry; and (*c*) depletion of albumin and other high-molecular-weight components that comprise most of the total protein mass in urine. Standard methods that have been applied for protein concentration—centrifugal ultrafiltration, acetone precipitation, acid precipitation, dye precipitation, ultracentrifugation, and lyophilization—generally have drawbacks of poor peptide recovery, poorly soluble pellets, or failure to remove salts *(6)*. We examined solid-phase extraction of urinary peptides, using a polymeric sorbent with a pore size that should exclude albumin and other proteins of similar or greater size.

Urine specimens were processed in 6-mL cartridges containing 500 mg of Strata$^{TM}$-X polymeric sorbent (Phenomenex) on a vacuum manifold. Pore size of the sorbent was specified by the manufacturer to be 91 Å, yielding a predicted size exclusion limit of ~20 000 Da. Cartridges were primed with 4 mL of methanol followed by 4 mL of 5 g/L acetic acid before addition of urine specimens, which had been acidified with acetic acid during collection to a pH of 4–5. After extraction of urine, cartridges were washed with 8 mL of 5 g/L acetic acid, and peptides were eluted with 3-mL steps of increasing acetonitrile concentration or with 600 mL/L acetonitrile–5 g/L acetic acid. Measurements of total protein and albumin to determine the amounts of proteins eluted from the cartridges were performed by standard methods (pyrogallol red and immunoturbidimetry, respectively) on a LX-20 analyzer (Beckman-Coulter). C-Peptide was measured by competitive immunoassay with an Immulite 2000 (Diagnostics Products Corp.). Eluates from extraction cartridges were analyzed either after evaporation under nitrogen to ~2 mL or directly.

Matrix-assisted laser desorption/ionization time-of-flight (MALDI TOF) mass spectrometry was performed with an Ultraflex TOF mass spectrometer (Bruker Daltonics) in a linear positive-ion mode. Specimens were applied manually to 384-position target plates as 1-μL aliquots between layers of matrix applied as 1-μL aliquots of 10 g/L sinapinic acid in 750 mL/L acetonitrile–250 mL/L water containing 10 g/L acetic acid. Data were summed for 300 laser pulses collected from 10 positions. Measurements of mass/charge (*m/z*) were by external calibration. Calibrators and sinapinic acid were purchased from Bruker Daltonics.

Solid-phase extraction of highly proteinuric urine (Table 1) served as good example of the size selectivity and binding capacity of the extraction cartridge. We loaded 10-mL aliquots of urine successively on a single cartridge and analyzed the eluates. The albumin concentrations of flow throughs were approximately the same as the initial